Investigating the role of intra- and extra-cellular modulators of the transcription factor NF-kappaB

By

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Submitted to the Department of Biology in partial fulfillment of the requirements for the degree of

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Abstract

The nuclear factor-kappa B (NF-κB) family of transcription factors play a pivotal role in the inflammatory and immune responses, as well as cell growth and survival. Since its discovery 25 years ago, NF-κB has emerged as one of the most intensely studied eukaryotic transcription factors, due to its pleiotropic functions and its inducible pattern of expression that is subject to multi-level regulation. Many modulatory events affect NF-kB function and thus to have a better understanding of the NF-κB pathway and its gene regulation will require additional studies of NF-κB's modulators.

The goal of this thesis was to investigate the role of intra- and extra-cellular modulators of NF-κB. To study how intracellular modulators affect NF-κB function, various genetic and biochemical approaches were used to identify potentially new NF-κB's interacting proteins. Two AGC family kinases, STK38 and STK38L, were chosen for further study. These two proteins were found to not only interact with NF-κB's p65 subunit but also act as its kinases in vitro. shRNA mediated knockdown combined with gene expression analysis revealed that STK38 and STK38L via their kinase function regulated p65 activity on some, but not all NF-κB-responsive inflammatory genes in response to TNFα stimulation. To study the role of extracellular modulators on the apoptotic behaviors of cells, a panel of human immortalized and cancer liver cell lines were pre-treated with pro-inflammatory cytokines TNFα and IL-1α followed by TRAIL, a potent inducer of cell death. This context-dependent approach revealed that TNFα and IL-1α had diverse effects on cell death behaviors and inhibiting the NF-κB pathway was enough to block pro-inflammatory cytokines-mediated pro-death or pro-survival outcomes. Chromatin immunoprecipitation followed by high throughput sequencing, realtime PCR and Western blotting were used to identify NF-κB's target genes involved in the cell death pathway. From these data, new regulatory mechanisms that affect cell death behaviors were proposed.

Dysregulation of NF-κB activation has been implicated in various pathologies including inflammatory diseases and cancer. The results described here represent progress toward understanding the NF-κB pathway and its gene regulation program. The more we understand this pathway, the better we are at targeting it for disease treatments.

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Chapter 1: Introduction

Discovered 25 years ago, the transcription factor nuclear factor-kappa B, also known as NF-κB is still one of the most studied proteins due to its pleiotropic functions and its involvement in inflammatory diseases and cancer (Ghosh and Hayden, 2008; Ghosh et al., 1998; Karin, 2009; Sen and Baltimore, 1986; Vallabhapurapu and Karin, 2009). NF-κB directly regulates a wide range of genes that control the inflammatory and immune responses, programmed cell death, cell proliferation and differentiation (Chen and Greene, 2004). The important role played by NF-κB proteins is illustrated not only by the diversity of genes that they regulate, but also by the large variety of stimuli that lead to their activation (Wan and Lenardo, 2010).

The Rel/NF-κB transcription factor family is comprised of several structurally-related proteins that exist in organisms from insects to humans (Barkett and Gilmore, 1999; Gonzalez-Crespo and Levine, 1994). The vertebrate family is expressed by five known NF-κB/REL genes, RELA, c-REL, RELB, NFKB1, and NFKB2 that give rise to seven protein products: RELA (p65), c-REL, RELB, p105/p50, and p100/p52 (p50 and p52 are the cleaved forms of p105 and p100, respectively). In cells, NF-κB proteins interact to form homo- and heterodimers, the most abundant of which is a p65/p50 heterodimer containing RELA and the cleaved form of the NFKB1 gene product. Dimerization is mediated by an N-terminal REL-homology domain (RHD) of ~300 amino acids that is present in all NF-κB proteins and is also responsible for binding to DNA and to the IκB family of NF-κB inhibitors (Hoffmann and Baltimore, 2006). All IκB proteins have a core domain, which consists of multiple ankryin repeats necessary for interactions with Rel proteins. The best-studied IκB protein is IκBα. The C-terminus of the pre-processed form of p105 and p100 also contains ankryin repeats, and thus can act to inhibit NF-κB in a similar manner like IκBs (Chen and Ghosh, 1999).

Activation of NF-κB is tightly controlled both in the cytoplasm and in the nucleus. While the early cytoplasmic events that lead to nuclear translocation of NF-κB have been studied in considerable detail, current understanding of NF-κB regulation does not fully explain its temporal, ligand or cell-type specificity. Emerging evidence suggests that posttranslational modification and recruitment of cofactors play a key role in determining the duration and strength of NF-κB nuclear activity as well as its gene expression program (Natoli and De Santa, 2006). Thus, to gain a better insight into the molecular mechanisms that mediate cell type- and stimulus-specific functions of this pleiotropic signaling system will require additional studies of the intra- and extra-cellular modulators of NF-κB.

1.1 Specific aims

In this thesis, I set out to investigate the role of intra- and extra-cellular modulators of NF-κB. The first aim examines the role of intracellular modulators of NF-κB. I first generated a knock-in cell line carrying an affinity purification tag. Then, using affinity purification followed by mass spectrometry, potentially new NF-κB's interacting proteins were identified. Two p65's binding protein STK38 and STK38L were selected for further study. Since these two proteins belong to the AGC kinase family (Hergovich et al., 2008), in vitro kinase assay was carried out to determine whether p65 is a substrate. Next, phospho-mass spectrometry was used to identify the phosphorylation sites. To study how STK38 and STK38 affect p65 function, an shRNA double knockdown cell line of STK38 and STK38L was generated. Gene expression microarray was performed to find NF-κB -dependent genes that were also coregulated by STK38 and

STK38L. Chromatin immunoprecipitation was performed to study how STK38 and STK38L affect p65 binding to certain gene promoters.

The second aim explores the role of extracellular modulators of NF-κB. A panel of immortalized and cancer liver cell lines were stimulated with pro-inflammatory cytokine TNFα or IL-1α for various amounts of time. To investigate how pretreatments of cells with TNFα or IL-1α affect their apoptotic responses, TRAIL was added to induce cell death following pro-inflammatory cytokine treatments. TNFα and IL-1α were observed to induce diverse apoptotic outcomes. To examine whether NF-κB is responsible for such outcomes, an NF-κB dominant negative inhibitor was introduced into cells. To identify genes activated by NF-κB in response to pro-inflammatory cytokine treatment, a chromatin immunoprecipitation followed by high throughput sequencing experiment was carried out followed by realtime PCR and Western blotting. This type of context-dependent approach allowed different NF-κB gene regulation programs to be examined, which can lead to identification of new regulatory mechanisms.

1.2 The canonical pathway

This pathway is activated by inflammatory cytokines such as TNFα and IL-1α, by engagement of T-cell receptor or by exposure to bacterial products such as LPS (Vallabhapurapu and Karin, 2009) (Figure 1.1A). The NF-κB dimers involved in this pathway are p65-p50 and c-Rel-p50 with the p65-p50 dimer being the most studied. In unstimulated cells, NF-κB proteins are sequestered in the cytoplasm due to their association with a family of inhibitor proteins called IκBs with IκBα and IκBβ being the most relevant (Baldwin, 1996; Verma et al., 1995). Upon ligand binding and activation of receptors, different types of adaptor molecules are recruited to

the receptors' intracellular domains (details on the adaptor's recruitment will be discussed in later sections), which results in the activation of an IκB kinase complex IKK (Li et al., 1999). This kinase when activated phosphorylates IκBα on two serine residues, S32 and S36, and thus marking this inhibitor for further modifications by ubiquitin enzymes and eventually degradation by the 26S proteosome. Once IκB is removed, NF-κB can freely translocate into the nucleus where it binds to gene promoters and regulate their expression.

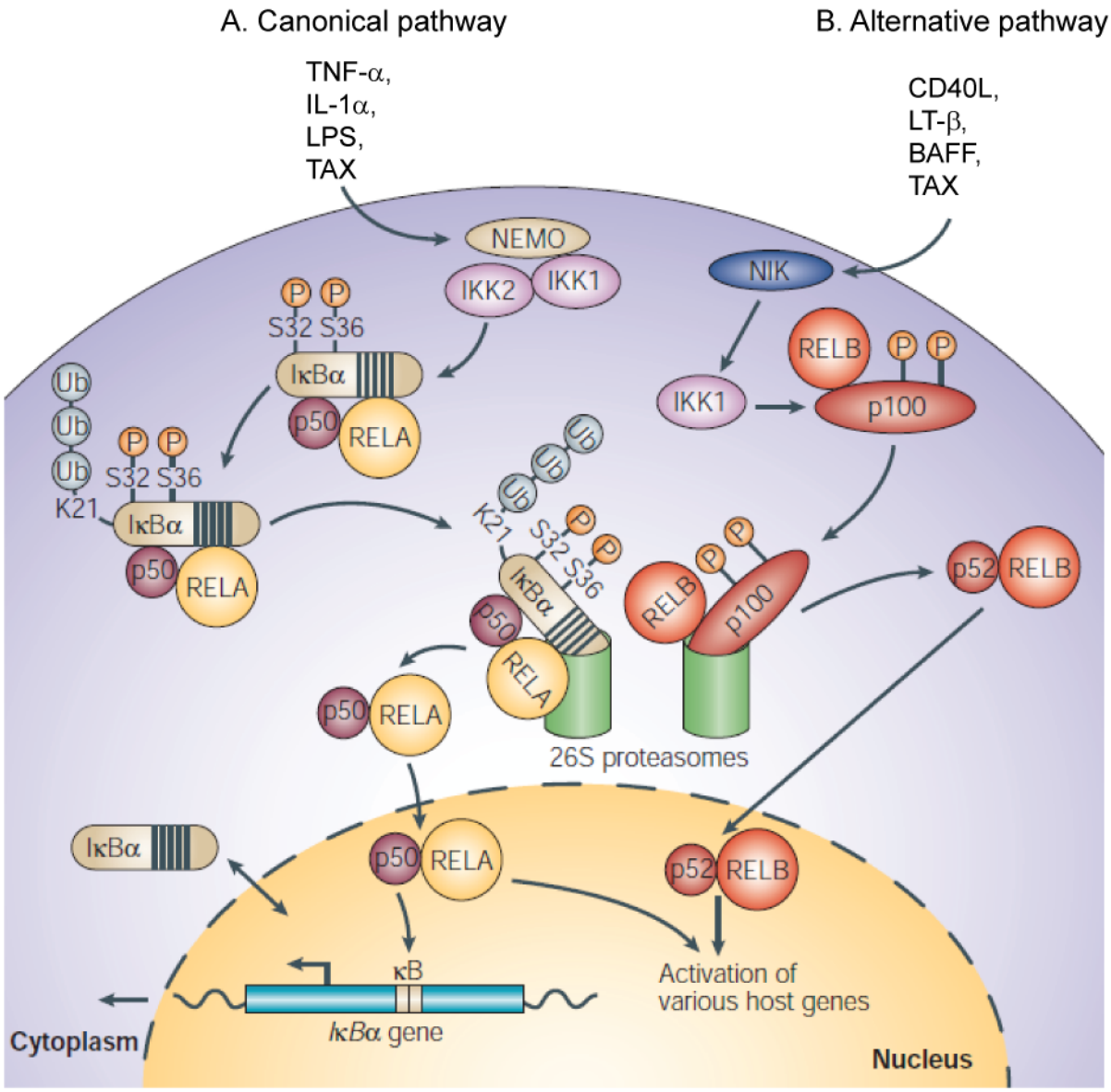

Figure 1.1 Activation of the NF-κB pathway (A)The canonical pathway is activated by inflammatory cytokines such as TNF-α and IL-1α or bacterial lipopolysacharide (LPS) or viral protein TAX. In unstimulated cells, NF-κB, primarily the RelA (p65)-p50 heterodimer, is sequestered in the cytoplasm by an NF-κB inhibitor protein IκB. Ligand binding leads to recruitment of adaptor proteins to the intracellular domain of receptors and activation of a kinase complex, IKK. IKK2 (IKK-β) of actiavted IKK phosphorylates IκBα and marks this inhibitor protein for proteosomal degradation. Degradation of IκBα frees NF-κB and allows it to enter the nucleus to activate gene transcription. (B) The alternative pathway is actiavted by stimuli including CD40 ligand (CD40L), lymphotoxin beta (LT-β), B cell–activating factor (BAFF). In unstimulated cells, NF-κB, primarily the RelB-p100 heterodimer, is sequestered in the cytoplasm. Ligand binding leads to recruitment of adaptor proteins to the intracellular domain of receptors and activation of the IKK1 upstream kinase called NIK. Activated IKK1 (IKK-α)

phosphorylates p100 and results in its partial degradation. The new NF-κB dimer of RelB and p52 (the partially degraded product of p100) translocates into the nucleus and activates gene transcription. Adapted from Chen and Greene, 2004.

1.3 The alternative pathway

This pathway is activated in response to a small subset of TNF family members, including CD40L (CD40 ligand), LTβ (lymphotoxin beta), BAFF (B cell–activating factor), RANKL (receptor activator of NF-κB ligand), and TWEAK (TNF-related weak inducer of apoptosis) (Claudio et al., 2002; Coope et al., 2002; Saitoh et al., 2003; Yilmaz et al., 2003) (Figure 1.1B). The main NF-κB dimer involved in this pathway is the p100-RelB heterodimer. Similar to the canonical pathway, in resting conditions this dimer is kept in the cytoplasm with the C terminus of p100 functioning like an IκB protein. An extracellular stimulation then leads to activation of a kinase called NIK (NF-κB inducing kinase), which is an upstream kinase of IKKα (Senftleben et al., 2001; Xiao et al., 2001). Activated IKKα phosphorylates the C terminus of p100, resulting in its partial degradation and the remaining protein p52 together with the bound RelB is liberated and can translocate into the nucleus and activate a gene transcription program.

1.4 Extracellular modulators of NF-κB

NF-κB has been shown to be activated by a variety of extracellular signals via several main groups of receptors including the TNF receptors family, the pattern-recognition Toll like receptors (TLR), the IL-1 receptors and the antigen receptors (Vallabhapurapu and Karin, 2009). These receptors can potentially activate not only different members of the NF-κB family but also other intracellular signaling and enzymatic proteins, which together results in the induction

of a unique gene transcription program. In the following sections, key and important molecules in each receptor-mediated NF-κB activation pathway will be described.

1.4.1 The TNFR pathway

TNFα is the prototypic member of a large family of trimeric cytokines and is one of the most potent activators of NF-κB. TNFα binds to two receptors TNFR1 and TNFR2. TNFR1 is present on most cell types and plays a much broader role in NF-κB activation (Chen and Goeddel, 2002). Upon ligand binding, TNFR1 forms a homotrimer complex which induces the recruitment of adapter proteins including TNFR1-associated death domain protein (TRADD), TNFR-associated factor 2 and 5 (TRAF2/5) and receptor interacting protein 1 (RIP1) to its cytoplasmic domain (Hsu et al., 1996; Micheau and Tschopp, 2003) (Figure 1.2). TRAF2 then recruits IKK to the receptor and also causes K63-linked ubiquitination of RIP1 (Ea et al., 2006; Meylan et al., 2004). Binding of IKKγ (NEMO) to ubiquitinated RIP1 stabilizes IKK interaction with the receptor complex, which facilitates its activation by either TGF-beta activated kinase 1 (TAK1) or MAPK kinase kinases 3 (MEKK3) (Blonska et al., 2004; Wang et al., 2001). Activated IKK phosphorylates IκBα at serine 32 and 36, resulting in its degradation by the proteosome. Degradation of IκBα frees the NF-κB p65-p50 heterodimer, allows it to migrate to the nucleus and regulate gene expression.

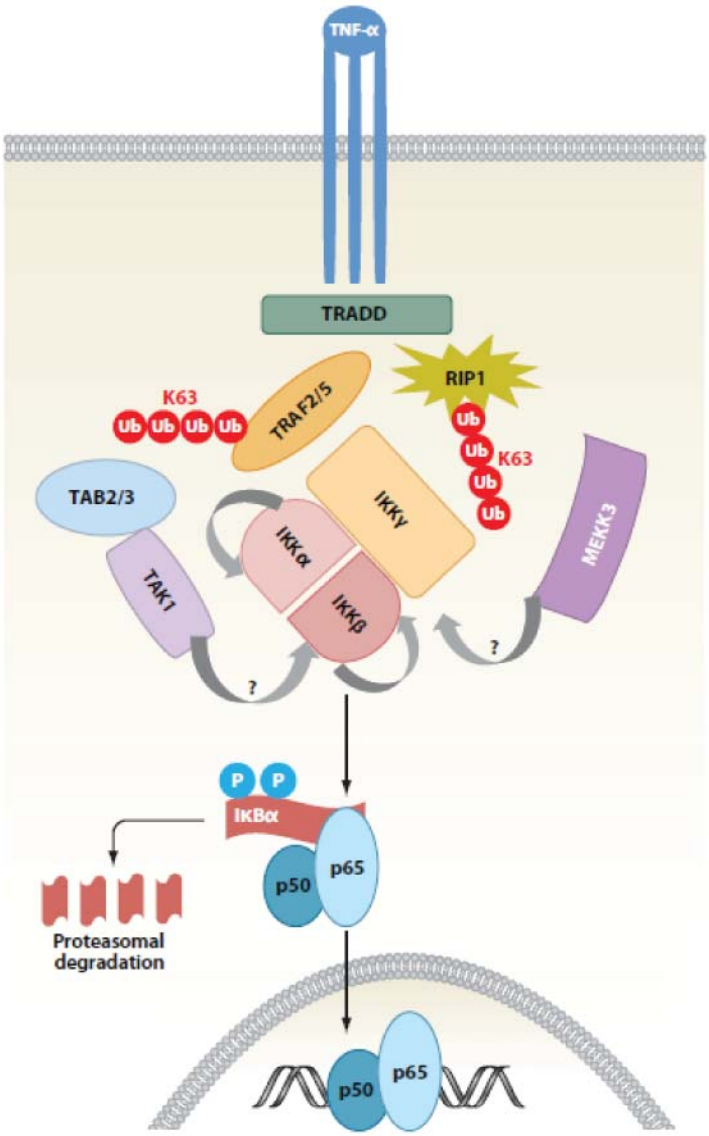

Figure 1.2 The TNFR pathway. Ligation of TNFR results in TRADD-dependent TRAF2/TRAF5 and RIP1 recruitment. TRAF2 causes K63-linked ubiquitination of RIP1 and also recruits IKK to the receptor complex. TAB2 and TAB3 interact with TRAF2 and TAK1, leading to TAK1 activation and phosphorylation of IKKβ. Alternatively, MEKK3, which is brought near the receptor complex presumably by RIP1 can also phosphorylate and activate IKK. Activated IKK phosphorylates IκBα at specific serine residues, leading to its proteasome-mediated degradation. Degradation of IκBα releases the NF-κB heterodimers and allows it to translocate into the nucleus and regulate gene expression. Adapted from Vallabhapurapu and Karin, 2009.

1.4.2 The TLR/IL-1 pathway

Members of the TLR/IL-1R family are potent inducers of the NF-κB canonical pathway. The cytoplasmic regions of TLR/IL-1R family proteins share a common motif called the Toll/Interleukin-1 receptor (TIR) domain (Kawai and Akira, 2007). Similar to TNFRs, ligation of TIR-containing receptors leads to recruitment of adapter proteins, ubiquitin ligases, and kinases to generate and transduce signals. Two adapter proteins, myeloid differentiation primary response gene 88 (MyD88) and TIR domain– containing adapter-inducing IFN-β (TRIF), play important roles in TLR/IL-1R signaling (Martin and Wesche, 2002). MyD88 recruits TRAF6 and IL-1R-associated kinase 1 (IRAK1), leading to self-ubiquitination of TRAF6 and recruitment of TAK1 binding protein 2/3 (TAB2/3), which in turn activate TAK1, an upstream kinase of IKKβ (Cao et al., 1996; Ishitani et al., 2003; Qian et al., 2001). Activated IKK causes phosphorylation and degradation of IκBα, resulting in NF-κB activation. Similar to MyD88, TRIF also recruits TRAF6, in addition to RIP1. TRAF6 and RIP1 together activate TAK1, culminating in IKK and NF-κB activation. In the case of TLR4 receptor, MyD88 and TRIF are recruited to these receptors via two bridging proteins TIR-adaptor protein (TIRAP for MyD88) and TRIF-related adaptor molecule (TRAM for TRIF) (Dunne et al., 2003; Yamamoto et al., 2003) (Figure 1.3).

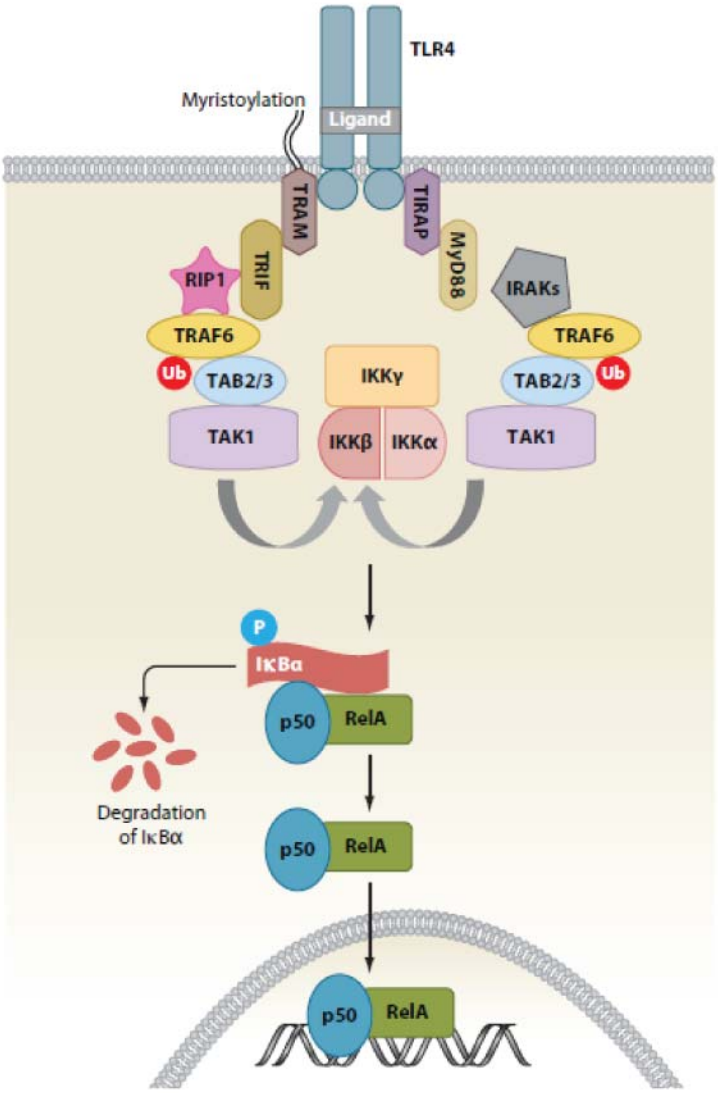

Figure 1.3 The TLR/IL-1 pathway. Ligand binding by TLRs results in the recruitment of receptor-specific adapters and induces activation of NF-κB. In the case of TLR4, LPS binding results in NF-κB activation via both TRIF- and MyD88-dependent pathways. TIRAP and TRAM serve as bridging factors to recruit MyD88 and TRIF, respectively. MyD88 recruits TRAF6 and members of the IRAK family, leading to oligomerization and self-ubiquitination of TRAF6 as well as recruitment of TAB2 and TAB3, which in turn activate TAK1. Activated TAK1 phosphorylates IKKβ, resulting in IκBα degradation and NF-κB activation. TRIF also recruits TRAF6. TRAF6 then activates TAK1, culminating in IKK and NF-κB activation in a manner similar to the MyD88-dependent pathway. In addition to recruiting TRAF6, TRIF also recruits RIP1, which might cooperate with TRAF6 to facilitate TAK1 activation. Adapted from Vallabhapurapu and Karin, 2009.

1.4.3 The TCR/BCR pathway

Stimulation of the T-cell and B-cell receptor (TCR/BCR) also results in IKK activation. The protein kinase C, PKCθ in T cells and PKCβ in B cells, plays a central role in recruiting intracellular signaling and adapter molecules to form spatially and temporally organized structures called supra-molecular activation clusters (SMACs) (Schulze-Luehrmann and Ghosh, 2006; Su et al., 2002; Sun et al., 2000). In TCR signaling, these SMACs include caspase recruitment domain membrane associated guanylate kinase protein1(CARMA1), B-cell lymphoma/leukemia 10 (BCL10), and mucosa-associated lymphoid tissue 1 (MALT1) (Weil and Israel, 2004) (Figure 1.4). The complex containing CARMA1, BCL10 and MALT1 facilitates the K63- polyubiquitination of IKKγ (NEMO), which results in activation of IKK and NF-κB. (Zhou et al., 2004).

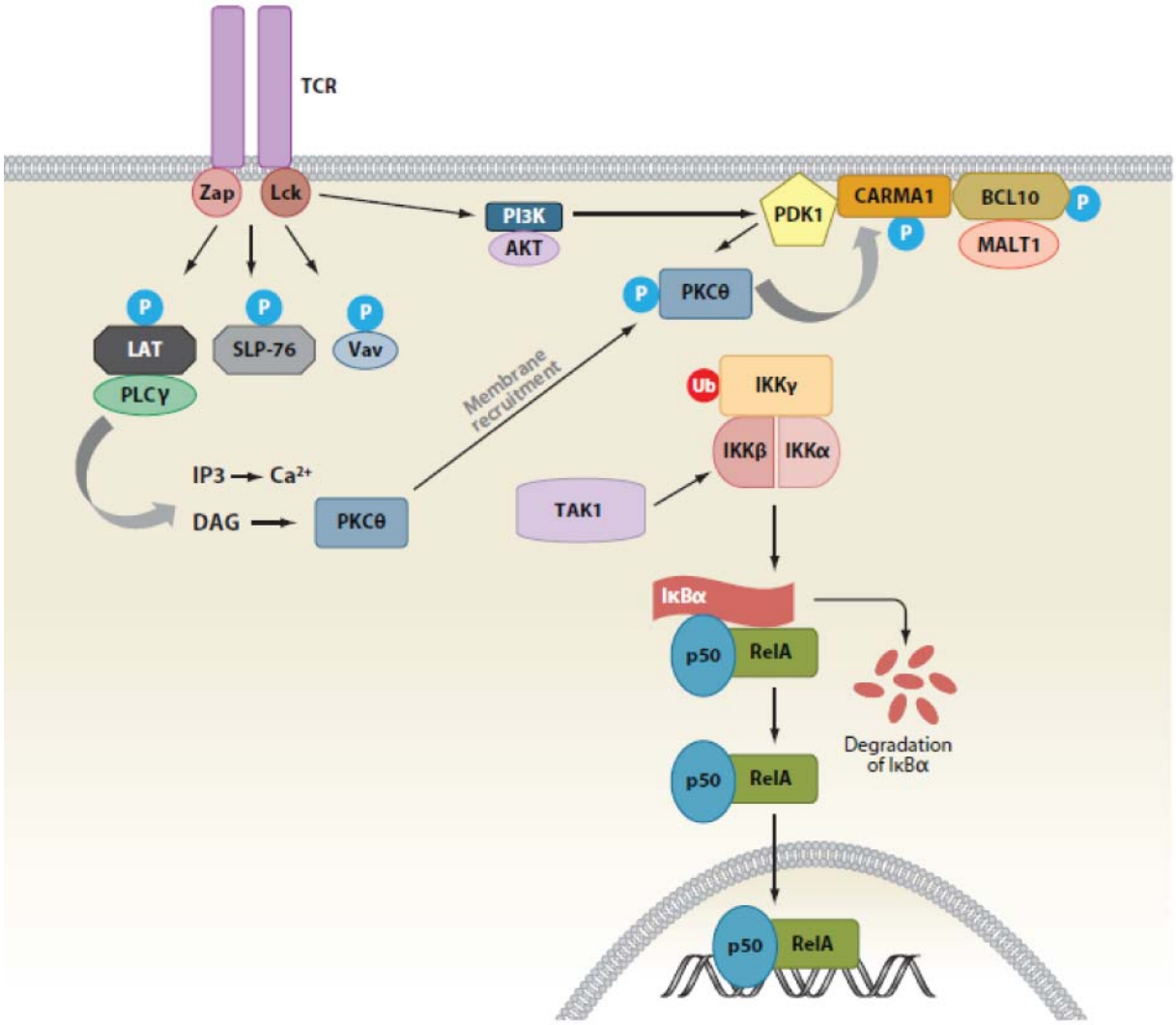

Figure 1.4 The TCR pathway. Stimulation of a TCR results in recruitment and activation of the Src (Lck) and Syk (Zap) family kinases. Zap phosphorylates adapter proteins LAT and SLP-76, leading to formation of a multimolecular complex containing PLCγ. Activation of PLCγ results in generation of IP3 and Ca2+, as well as DAG, which in turn stimulates PKCθ. Signals from TCR and CD28 costimulation result in activation of PI3K, which facilitates recruitment of PKCθ to the membrane. Phosphorylation of the phosphoinositides by PI3K leads to membrane recruitment of PDK1, which phosphorylates and activates PKCθ to control the recruitment of IKK and CARMA1 into the signaling complex. Phosphorylation of CARMA1 by PKCθ results in the recruitment of BCL10 and MALT1, leading to formation of a stable CBM complex. A poorly defined mechanism involving ubiquitination of IKKγ and activation of IKKβ presumably by TAK1 then leads to activation of IKK complex, which phosphorylates IκBα, resulting in its degradation and activation of NF-κB. Adapted from Vallabhapurapu and Karin, 2009.

1.5 Intracellular modulators of NF-κB

NF-κB is known to regulate hundreds of genes involved in many important cellular responses such as inflammation, migration, proliferation and apoptosis (Chen and Greene, 2004). So the obvious question is how a single transcription factor family can regulate so many different genes. Apparently, the early cytoplamsic events leading to the release and translocation of NF-κB into the nucleus are not sufficient to explain the complex nature of NF-κB biology. In the last decade or so, it has become clear that additional events including posttranslational modifications and recruitment of cofactors play important roles in the NF-κB-dependent gene regulation (Huang et al., 2010; Wan and Lenardo, 2010). In the following sections, known NF-κB's posttranslational modifications and coregulators will be described.

1.5.1 Posttranslational modifications

Emerging evidence suggests that like other pleiotropic transcription factors such as p53, NF-κB is also subject to a variety of posttranslational modifications and that these modifications affect the duration and strength of NF-κB activation as well as its transcriptional output. Most of the known posttranslational modifications including phosphorylation, acetylation and methylation are associated with the NF-κB's p65 subunit (Huang et al., 2010). The following sections will thus focus on describing posttranslational modifications of p65.

1.5.1.1 Phosphorylation

Like kinases, phosphorylation of a transcription factor provides a powerful mechanism for the rapid and reversible integration of intracellular signals that ultimately results in activation of gene expression (Hunter, 2000). p65 has been shown to be phosphorylated by many kinases at multiple residues including seven serines and three threonines (Chen and Greene, 2004) (Figure

1.5A). Phosphorylation of p65 can occur both in the cytoplasm and in the nucleus. Depending on the site of phosphorylation, the target gene, and the stimulus, phosphorylation can cause an increase or decrease in the level of transcription.

Phosphorylation of serine 276 was first identified to be mediated by the catalytic subunit of the protein kinase A (PKAc) in response to lipopolysaccharide (LPS) (Zhong et al., 1997). Other ligands including TNF-α- and TGF-β were later found to also be able to activate PKAc and induce PKAc-mediated phosphorylation of S276 (Ishinaga et al., 2007; Jamaluddin et al., 2007). Phosphorylation of S276 is believed to cause a conformational change in p65, which facilitates its binding to coregulators such as p300/CBP, leading to the overall increase in the transcriptional activity of NF-κB. S276 can be phosphorylated by other kinases including the mitogen- and stress-activated protein kinase-1 (MSK1) in response to TNF-α, IL-1β, respiratory syncytial virus (RSV) and Helicobacter pylori infection (Pathak et al., 2006; Reber et al., 2009; Vermeulen et al., 2003). While PKAc phosphorylates p65 in the cytoplasm, MSK1, on the other hand, phosphorylates p65 in the nucleus.

Another well-studied phosphorylation site of p65 is serine 536. This site can be modified by different kinases including IKKs, ribosomal subunit kinase-1 (RSK1), and TANK binding kinase (TBK1) under various conditions with different functional outcomes (Adli and Baldwin, 2006; Bohuslav et al., 2004; Buss et al., 2004b; Sakurai et al., 1999). The IKKs-mediated phosphorylation of S536 occurs in response to TNF-α, LPS, H. pylori and human T-lymphotrophic virus-1 (HTLV1)-encoded TAX protein. Phosphorylation of S536 was also shown to enhance the transcriptional activity of NF-κB either by increasing p65's binding with p300 and decreasing its binding with co-repressor SMRT (as in the case of IKK) or by lowering

p65's affinity for IκBα and decreasing IκBα-mediated nuclear export of NF-κB (as in the case of RSK1 and TBK1).

NF-κB's p65 can also be phosphorylated on serine 468. Interestingly, phosphorylation of S468 has different transcriptional outcomes depending on the cellular context. S468 is constitutively phosphorylated by GSK3β and this phosphorylation negatively affects the basal NF-κB activity (Buss et al., 2004a). In response to TNF-α and IL-1β, S468 is phosphorylated by IKKβ or IKKε, and phosphorylation of S468 by these kinases moderately reduces NF-κB activity via ubiquitin-mediated degradation of p65 (Geng et al., 2009; Mao et al., 2009; Schwabe and Sakurai, 2005). On the other hand, in response to T cell co-stimulation, IKKε was shown to phosphorylate p65's S468 and enhances the transcriptional activation of NF-κB (Mattioli et al., 2006).

Other residues involved in p65's phosphorylation and regulation include S205, S281, S311, S529, T254, T435 and T505. S311, T505 and S529 were shown to be phosphorylated by protein kinase C-zeta (PKCζ), Chk1 and casein kinase 2 (CK2), respectively (Duran et al., 2003; Rocha et al., 2005; Wang et al., 2000). S205, T254, S281 and T435 are also phospho-acceptor sites, but their kinases are not yet known. Phosphorylation of p65 on some of these residues (S205, S281, S311 and S529) enhances the transcriptional activity of selective NF-κB-dependent genes. On the contrary, T435 and T505's phosphorylation was shown to inhibit NF-κB activity (Huang et al., 2010).

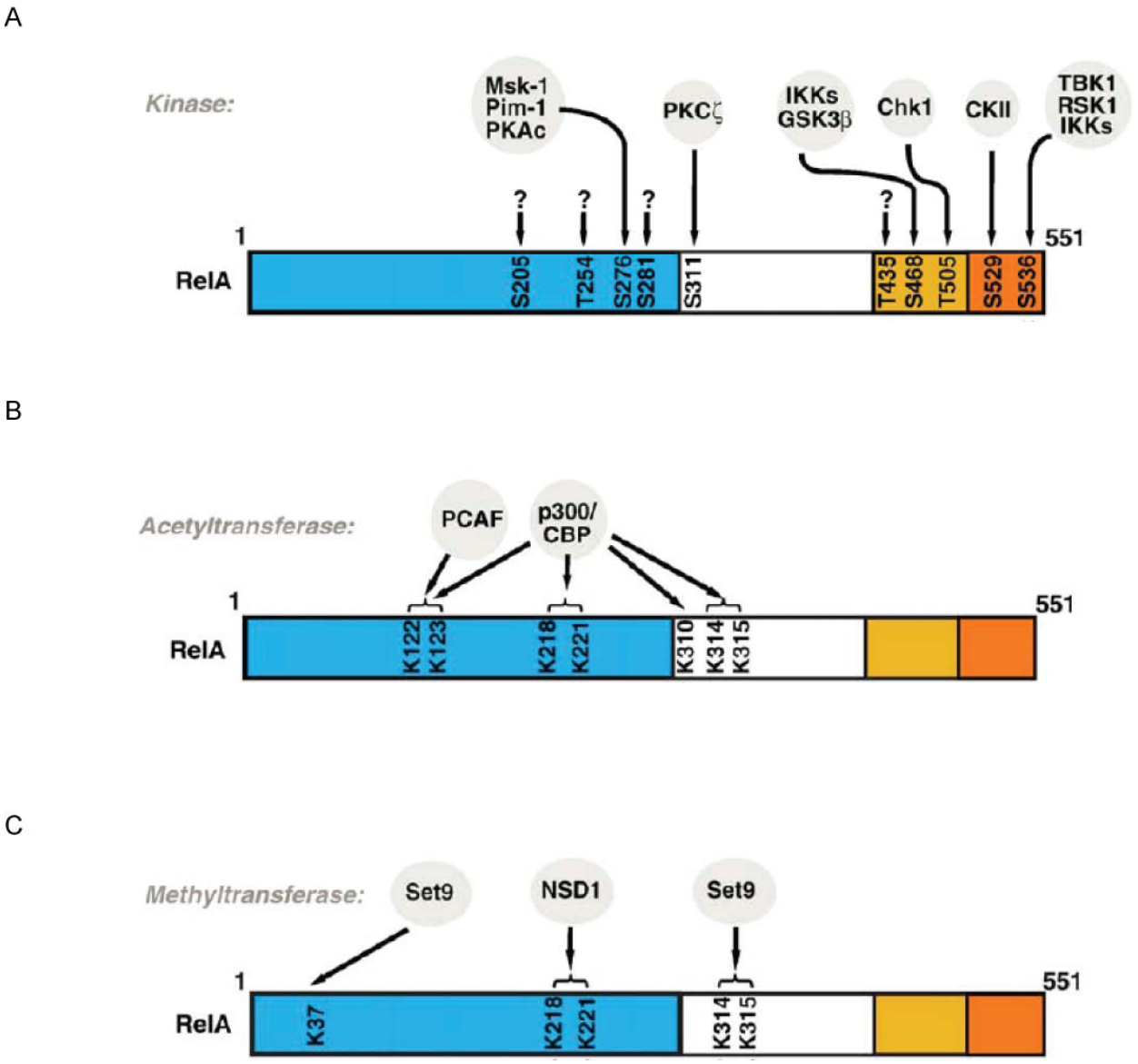

Figure 1.5 Posttranslational modifications of RelA (p65). (A) Known phosphorylation sites of RelA and their corresponding kinases. (B) Known acetylation sites of RelA and their corresponding acetyltransferases. (C) Known methylation sites of RelA and their corresponding methyltransferases. Question marks denote unknown enzymes. Adapted from Huang et al., 2010.

1.5.1.2 Acetylation

Acetylation is another important posttranslational modification of p65. Most acetylation events occur in the nucleus, where acetyltransferases mediate addition of the acetyl group to

lysine residues. Like phosphorylation, acetylation of p65 is a reversible process and plays a role in altering the DNA binding and transcriptional activity of NF-κB (Huang et al., 2010).

Seven lysine residues of p65 have been identified to be acetylated including lysine 122, 123, 218, 221, 310, 314 and 315 (Figure 1.5B). While most lysine residues are acetylated by p300/CBP, K122 and K123 can also be modified by PCAF (Kiernan et al., 2003). Acetylation of p65 on different lysines appears to affect the function of NF-κB differently. For example, acetylation at K221 enhances the DNA binding of NF-κB while acetylation at K122 and K123 reduces p65 binding to the κB promoters (Chen et al., 2002). While acetylation of K218 impairs NF-κB association with IκBα, acetylation of K310 does not affect DNA binding nor IκBα association. Interestingly, acetylation of K314 and K315 does not involve in regulating NF-κB shuttling or DNA binding, but appears to modulate the expression of specific sets of NF-κB target genes (Buerki et al., 2008).

1.5.1.3 Methylation

In addition to phosphorylation and acetylation, methylation has also emerged as an important modification for the regulation of nuclear NF-κB function. Most methylation events occur on lysine residues but some arginine can also be modified. Depending on the position as well as the state of methylation (mono-, di-, or tri-methylation), this can cause different effects on the function of NF-κB (Huang et al., 2010).

Several p65's lysine methyltransferases have been described (Figure 1.5C). These enzymes usually modify more than one lysine residue. For example, Set9 mono-methylates p65 at K37, K314 and K315 (Yang et al., 2009). Interestingly, while methylation of p65 by Set9 at K314 and K315 negatively affects the function of NF-κB by inducing a ubiquitin-mediated degradation of promoter-bound p65, methylation at K37 is implicated in the activation of a

subset of NF-κB target genes by stabilizing the binding of NF-κB to its enhancers (Ea and Baltimore, 2009). Another p65's methylase is NSD1. NSD1 mono-methylates K218 and di-methylates K221. Methylation of p65 by NSD1 enhances the transcriptional activity of NF-κB (Lu et al., 2010).

Besides lysine methyltransferases, arginine methyltransferases have also been implicated in regulating NF-κB functions. Three p65's arginine methyltransferases, PRMT1, PRMT2 and PRMT4 have been identified so far. It appears that not only is their enzymatic activity essential but also these arginine methyltransferases can bind and act as p65's cofactors (Covic et al., 2005; Ganesh et al., 2006).

1.5.2 Interacting proteins

Besides being regulated by posttranslational modifications, NF-κB's cofactors also play a crucial role in its stimulus- and promoter-specific transcriptional regulation. A vast array of coregulators, including coactivators and corepressors, have been identified (Wan and Lenardo, 2010). One example of such coactivators is the astrocyte elevated gene-1 (AEG-1) (Sarkar et al., 2008). AEG-1 interacts with NF-κB's p65 in a TNFα-dependent manner. Binding of AEG-1 helps facilitate the interaction of NF-κB with the basal transcriptional machinery at the promoter of the IL-8 gene and thus promoting its expression. An example of NF-κB's corepressors is the inhibitor of growth 4 (ING4) (Nozell et al., 2008). ING4 is recruited to NF-κB's promoters, which on the one hand results in inhibition of p65 phosphorylation and p300 binding, but on the other hand enhances the level of HDAC-1 at these promoters and thus negatively regulating NF-κB's transcriptional activity.

Interactions between NF-κB dimers and a variety of transcription factors have also been described. For example, some NF-κB -dependent genes induced by LPS also require the b-ZIP

transcription factor JunB (Krappmann et al., 2004). Cooperative interactions have also been shown between NF-κB and zinc-finger-containing transcription factors such as Sp1 and other b-ZIP factors such as C/EBP b (Perkins, 1997). The range of such interactions is likely to be large and this diversity plays a role in determining the heterogeneity of NF-κB -dependent gene expression.

1.6 Cellular functions of NF-κB

Studies over the past 25 years have led to identification of about a hundred genes directly regulated by NF-κB. These genes play important roles in many cellular pathways including immune development and function, cell proliferation and survival (Hoffmann and Baltimore, 2006). Examples of genes encoding inflammatory mediators are the pro-inflammatory cytokines TNFα, IL-1, and IL-12 and chemokines such as monocyte chemotactic protein -1 (MCP-1), interferon-inducible protein-10 (IP-10), and regulated upon activation, normal T-cell expressed and presumably secreted (RANTES). In addition, a large number of NF-κB -dependent genes contribute to the innate immune response such as the anti-microbial peptide b-defensin-2 or the C-reactive protein (Hoffmann and Baltimore, 2006). NF-κB also involves in regulating the expression of genes in the programmed cell death pathway. Most of the genes in this pathway, when upregulated, are known to have anti-apoptotic function and they work by interfering with c-Jun N-terminal kinase signaling (e.g. GADD45β), by preventing the accumulation of reactive oxygen species (e.g. MnSOD, FHC) and protecting the mitochondria (Bcl2 family members), or by inhibiting caspases (e.g. inhibitors of apoptosis (IAPs), cellular FLICE inhibitory protein (c-FLIP)) (De Smaele et al., 2001; LaCasse et al., 1998; Micheau et al., 2001; Pham et al., 2004). Other NF-κB's target genes involved in tissue remodeling (e.g. matrix-metalloproteinases) and

cell cycle regulatory proteins (e.g. cyclin D) have also been reported (Hinz et al., 1999; Okamoto et al., 2004).

1.7 Physiological roles of NF-κB

At the tissue level, NF-κB plays important roles in the development and differentiation of the immune system, in the dynamic regulation of local and systemic immune activity as well as in the development and survival of the liver. The following sections will provide details on this aspect of NF-κB.

1.7.1 Lymphocyte biology

Mice knock-out studies have found p65 and c-Rel to be critical for lymphocyte development and function (Horwitz et al., 1997; Kontgen et al., 1995). While cRel-deficient mice have normal numbers of many lymphocyte subclasses, mature lymphocytes, however, fail to activate certain pathways such as B- and T-cell proliferation and isotype switching. On the other hand, mice lacking p65 show embryonic lethality during embryonic day E14-15. The canonical NF-κB pathway plays an important role in providing survival and proliferative signals to lymphocytes, thus facilitating early B- and T-cell development (Siebenlist et al., 2005). In addition, this pathway is also required for the efficient development of unique T-cell subpopulations, such as natural killer T cells and CD4+CD25+ regulatory T cells (Schmidt-Supprian et al., 2004).

1.7.2 Secondary lymphoid organ development

Lymphocyte development also requires proper functioning of secondary lymphoid organs. Both canonical and non-canonical pathways are important for the secondary lymphoid

organogenesis as mice lacking p65, RelB, p50 or p52 are deficient in peripheral lymph nodes (Alcamo et al., 2002; Lo et al., 2006; Yilmaz et al., 2003). Lack of NF-κB activity in stromal, epithelial, or dendritic cell populations in thymus and spleen also causes developmental defects in the B- and T-cell compartments, which can result in defective deletion of auto-reactive T cells and establishment of central tolerance (Weih and Caamano, 2003).

1.7.3 Role of NF-κB in liver development and survival

NF-κB has an important role in liver physiology and function. p65-deficient mice show embryonic lethality during embryonic day E14-15 due to massive apoptosis in the fetal liver due to TNFα exposure (Horwitz et al., 1997). The hepato-protective role of NF-κB is due to its ability to activate genes that can prevent TNFα induced cell death (Sun and Karin, 2008). In hepatocytes, the most critical anti-apoptotic protein induced by NF-κB appears to be c-FLIP (Chang et al., 2006). FLIP inhibits apoptosis by interfering with caspase-8 activation (Micheau and Tschopp, 2003). In addition, induction of NF-κB -dependent genes encoding antioxidants such as SOD2 and ferritin heavy chain prevents the accumulation of reactive oxygen species, which can cause both apoptosis and necrosis (Kamata et al., 2005; Pham et al., 2004).

1.8 NF-κB in diseases

Aberrant NF-κB regulation has been observed in various disease including chronic inflammation and different types of cancers. High levels of active nuclear NF-κB are found in the affected organs of patients with rheumatoid arthritis, asthma, inflammatory bowel diseases, liver fibrosis and cirrhosis (Chen and Greene, 2004; Sun and Karin, 2008). Also, because of its role in regulating expression of many important genes, NF-κB is implicated in malignancy

processes including tumor cell proliferation through its ability to induce proto-oncogenes such as cyclin D1 and c-Myc, metastasis through expression of cellular adhesion molecules and matrix metalloproteinases; angiogenesis through regulation of vascular endothelial growth factor; and cell immortality through regulating telomerases (Perkins and Gilmore, 2006). Constitutively active forms of NF-κB are frequently detected in many cancers including both solid and hematopoietic tumors (Baud and Karin, 2009).

It is believed that NF-κB and the NF-κB pathway play a key role linking inflammation and tumor development (Karin, 2009). In most tumors, NF-κB is activated not due to intrinsic mutations but in response to inflammatory stimuli originating from the microenvironment. Mutations that endow RelA, c-Rel, or other NF-κB proteins with transforming activity are rare and mainly limited to lymphoid malignancies. A good example of inflammation-linked cancers is hepatocellular carcinoma (HCC), the most common form of liver cancer. Most HCC develops in the context of chronic hepatitis caused by either HBV or HCV viral infection (Sun and Karin, 2008).

1.9 Targeting NF-κB and its pathway in cancer therapy

NF-κB transcription factors play a key role in many physiological processes, such as innate and adaptive immune responses, cell proliferation, cell death, and inflammation. Aberrant regulation of NF-κB and its signaling pathways have been implicated in cancer development and progression, as well as in resistance to chemo- and radiotherapy.

Drugs that block NF-κB are currently in clinical trials with promising results. In murine xenograft models, proteasome inhibitors were shown to have significant anti-tumor activity and in squamous cell carcinoma tumor models, another inhibitor, PS-341, inhibits cell survival,

tumor growth and angiogenesis (Richardson et al., 2004; Yin et al., 2005). Most proteasome inhibitors work by blocking NF-κB nuclear localization through inhibition of IκBα degradation and processing of the p105 NF-κB precursor. However, as the major proteolytic system in eukaryotic cells, the ubiquitin–proteasome pathway controls the breakdown of many cellular proteins, thus inhibiting this pathway will affect other physiological processes. Other molecules have been shown to inhibit NF-κB including a peptide that blocks the interaction of IKKγ with the catalytic subunits of IKK, the non-steroidal anti-inflammatory drug (NSAID) sulindac, cyclopentenone prostaglandins, arsenic trioxide, thalidomide, a variety of anti-oxidants, natural products such as parthenolide and resveratrol, and pharmaceutically developed synthetic small-molecule inhibitors of the IKK complex (Baldwin, 2001; Kapahi et al., 2000; Keifer et al., 2001; Yamamoto and Gaynor, 2001). Specially, resveratrol was shown to induce apoptosis of Ras-transformed cells, and sulindac also induced cell death in certain transformed cells. Arsenic trioxide, which directly modifies a critical cysteine of IKK, has shown efficacy in several hematological malignancies. Another IKK inhibitor, PS-1145, inhibits multiple myeloma cell proliferation but not as effectively as the proteasome inhibitor PS-341 (Hideshima et al., 2002). Overall, these data underscore the potential relevance of targeting NF-κB in cancer by blocking different constituents in the NF-κB signal-transduction pathway. However, one drawback all these inhibitors suffer is selectivity as many of them not only block NF-κB but also affect other cellular pathways as well.

1.10 Toward a better understanding of the NF-κB gene regulation

NF-κB has emerged as a central player in a variety of physiological functions. It was initially thought that each NF-κB -inducing stimulus is capable of activating a specific gene

expression program by causing the degradation of a specific IκB protein, leading to the release of a specific NF-κB dimer that is capable of binding a subset of κB sites. However, there is little functional evidence for specificity due to IκB isoform-specific degradation by a particular stimulus or due to interaction between different NF-κB isoforms and κB sites. In fact, NF-κB dimers display overlapping κB-site recognition properties (Hoffmann and Baltimore, 2006). So how do cells achieve cell type-, stimulus and time-dependent responses employing a single signaling pathway? The NF-κB family of transcription factors provides a number of interesting paradigms for combinatorial control of gene regulation (Natoli and De Santa, 2006). Accumulating evidence indicates that specificity in NF-κB-regulated gene expression is generated at multiple levels including at the receptor level where each receptor can activate a specific signaling network and at the promoter level where each promoter only allows NF-κB with proper posttranslational modifications or cofactor recruitment to bind.

Alternative NF-κB stimuli can induce different sets of NF-κB-dependent genes and thus producing distinct biological responses. For example, while NF-κB activity induced by TNFα is rapidly attenuated due to the negative feedback mechanism involving the synthesis of its inhibitor IκB, NF-κB activity induced by LPS is sustained due to an autocrine loop of LPS-dependent TNFα induction and secretion. In addition, like many transcription factors, NF-κB is subject to posttranslational modifications. These modifications including phosphorylation, acetylation and methylation provide additional layers of regulation of NF-κB activity and its selective induction of target genes. Also, besides binding key players in its activation pathway, NF-κB has been shown to interact with many cofactors and these proteins can influence NF-κB activity in different ways. They can act as coactivitors to enhance NF-κB transcriptional activity or as co-repressors to suppress its activity. Finally, synergistic interaction between NF-κB and

partner transcription factors can also play a role in shaping specific aspects of the NF-κB response, such as stimulus- and tissue-specific expression of target genes. In the following chapters, studies of some of these NF-κB regulatory events are described.

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Chapter 2: The STK38 and STK38L kinases bind p65/RELA and play a role in NF-κB dependent transcription of inflammatory genes

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2.1 INTRODUCTION

Since its discovery more than 20 years ago (Sen and Baltimore, 1986), nuclear factor-kappa B (NF-κB) has become one of the most intensively studied eukaryotic transcription factors. In mammalian cells, NF-κB has diverse functions in regulating inflammation, immunity, apoptosis and proliferation (Baldwin, 1996; Hayden and Ghosh, 2008; Karin et al., 2002; Schmitz et al., 2004).and is expressed by five known NF-κB/REL genes, RELA, c-REL, RELB, NFKB1, and NFKB2, that give rise to seven protein products: RELA (p65), c-REL, RELB p105/p50, and p100/p52, (p50 and p52 are the cleaved forms of p105 and p100, respectively). In cells, NF-κB proteins interact to form homo- and heterodimers, the most abundant of which is a p65/p50 heterodimer containing RELA and the cleaved form of the NFKB1 gene product (Hayden and Ghosh, 2004). Dimerization is mediated by an N-terminal REL-homology domain (RHD) of ~300 amino acids that is present in all NF-κB proteins and is also responsible for binding to DNA and to the IκB family of NF-κB inhibitors (Hacker and Karin, 2006).

NF-κB is activated by a variety of stimuli including pro-inflammatory cytokines, growth factors, DNA-damaging agents and viral proteins (Hayden and Ghosh, 2008; Hoffmann and Baltimore, 2006). In the absence of these stimuli, NF-κB dimers are sequestered in the cytoplasm through association with IκBs. NF-κB activation via the canonical pathway involves phosphorylation of IκB by IκB kinase (IKK), a modification that marks IκB for ubiquitin-mediated degradation by the proteosome. This liberates the p65/p50 dimer from its inhibited state, allowing translocation into the nucleus and subsequent binding to the promoters of genes containing κB elements (Perkins and Gilmore, 2006). Analysis of promoters regulated by NF-κB has shown that κB sites exhibit a loose consensus sequence conforming to the motif $G_{-5}G_{-4}G_{-3}R_{-1}N_{-1}N_0Y_{+1}Y_{+2}C_{+3}C_{+4}$ (where R is purine, Y is pyrimidine and N is any base) (Lenardo and

Baltimore, 1989). Thousands of matches to this consensus are found in human and chimpanzee genomes (Kasowski et al., 2010) but the κB sequences actually involved in gene expression appear to be highly conserved between mouse and human (Leung et al., 2004) implying that the consensus sequence captures only part of the information controlling NF-κB binding selectivity.

The early cytoplasmic events that lead to nuclear translocation of NF-κB have been studied in considerable detail but current understanding of NF-κB regulation does not fully explain its temporal, ligand and cell-type specificity. In addition to being modified by multiple cytosolic kinases, events downstream of NF-κB nuclear transport such as the recruitment of cofactors control transcriptional activity (Chen and Greene, 2004; Wan and Lenardo, 2010). Initially, it was thought that the composition of NF-κB dimers determined which promoter sequences would be bound but it was subsequently shown that different dimers bind κB sites with little differential selectivity (Natoli and De Santa, 2006). In addition, subunits in NF-κB hetero- and homo-dimers can exchange with the unbound nuclear pool at a single promoter site during the course of a response (Saccani et al., 2003). It now seems that κB sequences specify which cofactors can form productive interactions with DNA-bound NF-κB and that it is these co-factors that are largely responsible for target selectivity (Leung et al., 2004). For example, the astrocyte elevated gene-1 (AEG-1) protein acts as an NF-κB co-activator for expression of the interleukin 8 gene and the inhibitor of growth 4 protein (ING4;, which binds p65 directly) acts as an NF-κB corepressor (Nozell et al., 2008; Sarkar et al., 2008).

Several types of post-translational modification have been identified as regulators of NF-κB activity. In the case of p65, phosphorylation, acetylation and methylation alter the efficiency of binding to DNA and association with transcriptional coregulators. For examples, p65 phosphorylation at serine 276 by PKAc or MSK1, or at serine 311 by PKCζ enhances p65

binding to CBP and p300, components of the basic transcriptional machinery (Duran et al., 2003; Vermeulen et al., 2003; Zhong et al., 1997). Overall, nine sites of serine/threonine phosphorylation on p65 have been reported to date. Multiple kinases modify these sites in response to a wide variety of stimuli and in some cases, a single kinase phosphorylates multiple p65 sites (Chen and Greene, 2004; Huang et al., 2010). For example, p65-S276 is phosphorylated in response to TNF-α by the catalytic subunit of protein kinase A (PKAc) or in the nucleus by mitogen- and stress-activated protein kinase-1 (MSK-1) or by the serine/threonine-protein kinase Pim-1 (Nihira et al., 2010; Vermeulen et al., 2003; Zhong et al., 1997). The IKKβ kinase phosphorylates p65 on residues S468 and S536, which results in inhibition or enhancement of gene expression depending on cellular context (Buss et al., 2004; Schwabe and Sakurai, 2005).

Given the complexity of NF-κB biology, a full understanding of its regulation will require studying proteins that associate with and modify its subunits and determining the consequences of binding and modification for transcriptional activity. Here we use affinity purification and mass spectrometry of epitope-tagged p65 expressed at its endogenous locus to identify potential NF-κB binding partners. Among the novel interacting partners arising from this analysis we focus on STK38 and STK38L (serine-threonine kinase 38 and 38-like), members of the AGC kinase family. These kinases are highly conserved from yeast to humans and have been implicated in diverse cellular processes such as mitotic exit, cytokinesis, centrosome duplication and apoptosis (Hergovich et al., 2008; Hergovich et al., 2006; Vichalkovski et al., 2008). Substrates of STK38 and STK38L in mammalian cells have been largely unexplored but we show here that the kinases can phosphorylate p65 in vitro and that their kinase activity is important for induction of multiple NF-κB-dependent inflammatory genes including TNF and

LTB. Our results suggest that STK38 and STK38L are new binding partners, modifiers and co-activators of NF-κB.

2.2 RESULTS

2.2.1 STK38/STK38L interact with the p65 subunit of NF-κB

To identify proteins that interact with p65, a FLAG-HA tandem affinity tag was knocked into the 3' end of RELA gene in an HCT116 colorectal cancer cell line using an adeno-associated virus protocol developed in the Vogelstein and Bunz laboratories (Kohli et al., 2004; Topaloglu et al., 2005). The knock-in construct and approach to identifying positive clones is shown in Figure 2.1. Affinity purification was performed on anti-FLAG resins using whole-cell extracts from cells expressing FLAG-HA-RELA. Eluted proteins were then subjected to liquid chromatography- mass spectrometry (LC-MS) which resulted in the identification in MS spectra multiple well characterized p65 interacting-proteins (p50, p52, RelB, c-Rel, IκB isoforms, and multiple 14-3-3 proteins (Bouwmeester et al., 2004)), thus validating our tagging and purification strategy (we note that this strategy does not distinguish between direct and indirect p65 interactors). Both untreated and TNF-α treated cells were examined, but few differences in the set of bound proteins was observed. However, LC-MS analysis was not carried out in a manner that would allow samples to be compared for quantitative rather than qualitative differences in the spectrum of bound proteins; our approach was focused on obtaining the longest list of candidate binding partners.

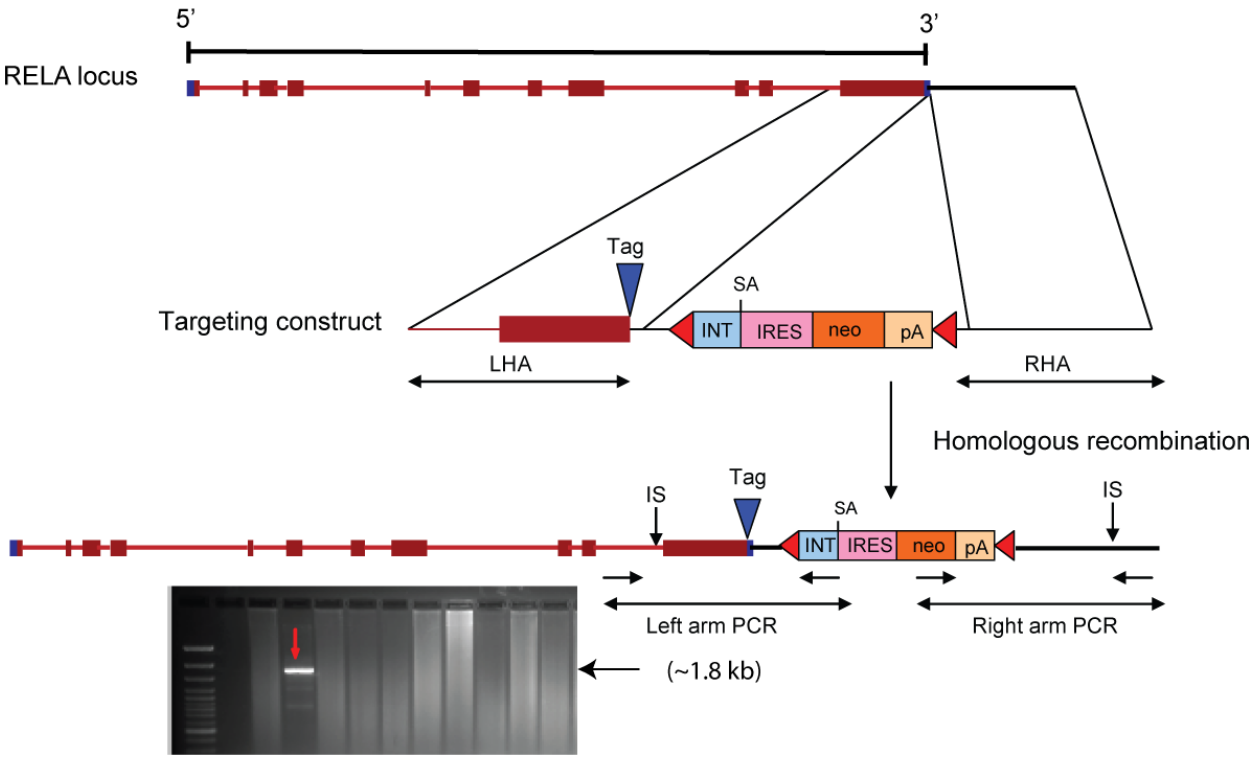

Figure 2.1 Generation of a knock-in cell line expressing tagged p65/RELA. (A) Design of knock-in construct based on adeno-associated virus methods from the Vogelstein and Bunz laboratories (Rago et al., 2007). A tandem affinity FLAG-HA tag (labeled "Tag ") was designed to be inserted at the C-terminus of p65. Abbreviations: LHA/RHA: Left/right homology arm. SA: splice acceptor; INT: intron; IRES: internal ribosomal entry site; neo: neomycin resistance gene; pA: poly A. IS: intergration site. Inset image is a representative agarose gel from PCR screening for clones in which homologous recombination had taken place. PCR primers were specific for the left arm or right arm as indicated. Positive clones were infected with adenovirus carrying Cre-recombinase expression vector to remove the neo selection cassette and ensure correct p65 expression.

Among the putative p65 binding proteins identified by MS, four previously unreported proteins were reproducibly isolated in our affinity purification procedure: CCT6A (T-complex protein 1, zeta subunit), STK38 (Serine/threonine-protein kinase 38, or NDR1), STK38L (Serine/threonine-protein kinase 38-like, or NDR2), SERBP1 (Plasminogen activator inhibitor 1 RNA-binding protein). We also identified PRMT1 (Protein arginine N-methyltransferase 1), which was shown during the course of our work to bind p65 and regulate transactivation (Hassa

et al., 2008). An extended list of candidate interacting proteins is presented in supplemental Table-S-2.1. cDNAs encoding each of the four new proteins as well as PRMT1 were cloned into vectors carrying a tandem calmodulin/streptavidin binding peptide affinity tag (CBP-SBP derived from Stratagene's pNTAP vectors) to allow affinity isolation and a StrepTag II tag to allow detection by Western blotting (Figure 2.2A). In the case of proteins whose molecular weights were similar to that of p65 (CCT6A, STK38, STK38L and SERBP1), an ~30kD N-terminal GFP tag was added to facilitate separation on SDS PAGE gels. p65 and tagged binding proteins were independently transiently transfected into 293T cells, lysates containing over-expressed p65 or candidate interacting proteins were mixed and affinity isolations were performed using resin carrying crosslinked streptavidin. Under these conditions we observed that all five proteins bound to p65 with three, STK38, STK38L and CCT6A, binding to a greater extent than the others (Figure 2.2B). When we repeated affinity isolations under more stringent conditions in which only the tagged interactors were over-expressed and p65 was present at endogenous levels, binding to p65 was most convincing for STK38 and STK38L (Figure 2.2C). We therefore concentrated our analysis on STK38 and STK38L (referred to as STK38/38L below) without ruling out the possibility that the other proteins are also able to associate with p65. Moreover, when we compared levels of ectopically-expressed and tagged STK38/38L bound to p65 in HCT116 before and after treatment with 100 ng/ml TNF-α for 3 and 5 hours (Figure 2.2D), no significant differences were observed. We conclude that STK38 and STK38L bind to p65 independent of stimulation with TNF-α.

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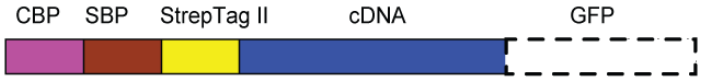
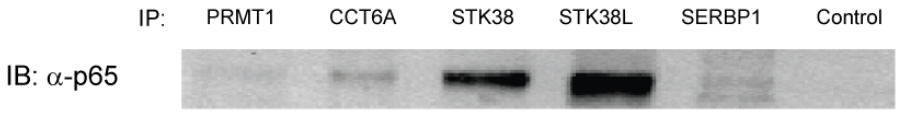

CBP
SBP
StrepTag II
cDNA
GFP

B


IP:
PRMT1
CCT6A
STK38
STK38L
SERBP1
Control
IB: α-p65

C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PRMT1
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STK38
STK38L
SERBP1
Control
IB: α-p65

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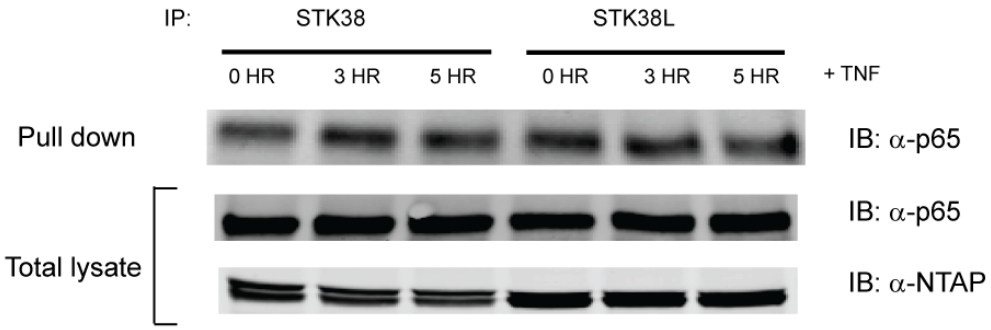
IP:
STK38
STK38L
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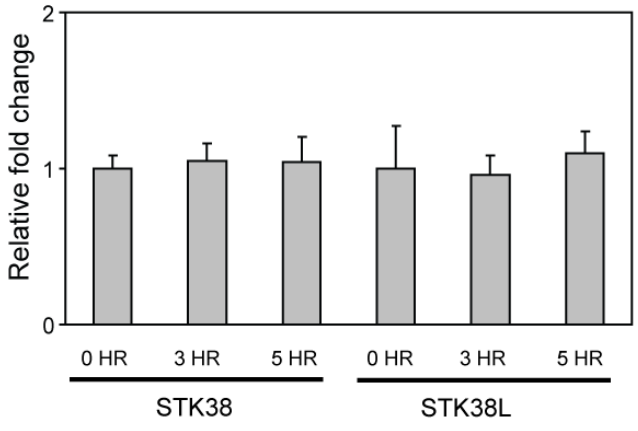
3 HR
5 HR
+ TNF
Pull down
IB: α-p65
Total lysate
IB: α-p65
IB: α-NTAP


Relative fold change
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0 HR
3 HR
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0 HR
3 HR
5 HR
STK38
STK38L

Figure 2.2 Verification of p65 binding proteins by co-precipitation. (A) Tagging strategy for confirming p65 binding by proteins identified as interactors by mass spectrometry. cDNAs encoding candidate p65-binding proteins were cloned into a pNTAP vector engineered to contain an N-termimal tandem affinity tag comprising a camodulin binding peptide (CBP; pink), a Streptavidin binding peptide (SBP; red), and a StrepTag II (yellow). In the case of proteins whose molecular weights were similar to that of p65, GFP was inserted at the C-terminus to increase apparent mass by ~30 kD. (B) Reciprocal immunoprecipitation was performed where both candidate proteins and p65 were ectopically-expressed in 293T cells and Strep resin was used to pull down the candidate proteins, control contains only p65 and resin. The same amount of p65-containing lysates was added in each IP sample. Abbreviations are as follows: PRMT1, protein arginine N-methyltransferase 1; CCT6A, T-complex protein 1, zeta subunit; STK38, serine/threonine-protein kinase 38 (or NDR1); STK38L, serine/threonine-protein kinase 38-like (or NDR2); SERBP1, plasminogen activator inhibitor 1 RNA-binding protein. CCT6A, STK38, STK38L and SERBP1 constructs include GFP. (C) Reciprocal immunoprecipitation as in (B) in which only candidate proteins were overexpressed in 293T cells while p65, expressed at endogenous levels, was purified from HCT116 cells. Lysates containing an overexpressed candidate protein and endogenous p65 were mixed prior to immunoprecipitation, control contains only endogenous p65 and resin. (D) Immunoprecipitation as in (C), but using STK38 and STK38L as bait proteins and p65 from cells treated with 100 ng/mL TNF-α for various times, as indicated. Lower plot quantifies the level of bound, normalized to the amount at 0 hr. (that is, in the absence of TNF-α). Error bars represent the standard deviation of the mean of biological duplicates.

2.2.2 STK38/STK38L phosphorylate p65 in vitro

To determine whether STK38 and STK38L phosphorylate p65, we performed in vitro kinase assays using enzymes and substrates isolated from normally growing 293T cells (kinases were isolated by affinity purification on Streptavidin resin and HA-tagged p65 was immuno-purified using anti-HA resin). p65 substrate was added at about twice the level of STK38/38L kinases (in mass terms) based on protein quantification using Coomassie stained SDS-PAGE gels. We observed significant auto- and trans phosphorylation of STK38, STK38L and p65; levels of radiolabel incorporation did not change significantly when kinases were purified from untreated cells or cells treated with either TNF-α or insulin for 6 hours (see supplemental Figure S-2.1). To demonstrate that the vitro kinase assays selectively measured STK38/38L activity, we generated STK38-K118A and STK38L-K119A mutants carrying lysine-to-alanine changes in the

catalytic site that rendered the kinases inactive. When in vitro kinases assays were performed with affinity purified STK38-K118A or STK38L-K119A, no increase in p65 phosphorylation was observed relative to negative control reactions (compare lanes 2, 4 and 5, Figure 2.3A); residual phopshorylation in control samples was likely due to low levels of contaminating kinases that co-purified with the p65 substrate.

To identify the sites at which STK38/38L modifies p65, we performed in vitro kinase assays followed by phospho-mass spectrometry. This analysis showed T308, S311 and S356 to be high certainty sites for phospho-addition, and S319, S338 and T458 to be less certain sites; no significant differences in the pattern of modification were observed between STK38 or STK38L. We mutated T308, S311 and S356 individually to alanine and repeated the vitro kinase assay. In each case we observed a roughly two-fold reduction in the levels of phosphorylated p65 with no change in the level of autophosphorylated kinase (which therefore serves as an internal control; Figure 2.3B). A double p65-S311A/ S356A mutant was not obviously less highly modified than single mutants and examination of mass spectrometry data did not uncover peptides doubly phosphorylated on T308 and S311. We conclude that p65 is phosphorylated in vitro by STK38/38L on T308, S311 and S356, probably at the level of one phosphorylation event per p65 molecule and that other residues are also modified at a lower stoichiometry. We speculate that when preferred phosphorylation sites are mutated, other residues become substrates. This is commonly observed for kinases that bind to their substrates (e.g. (Jeffery et al., 2001)).

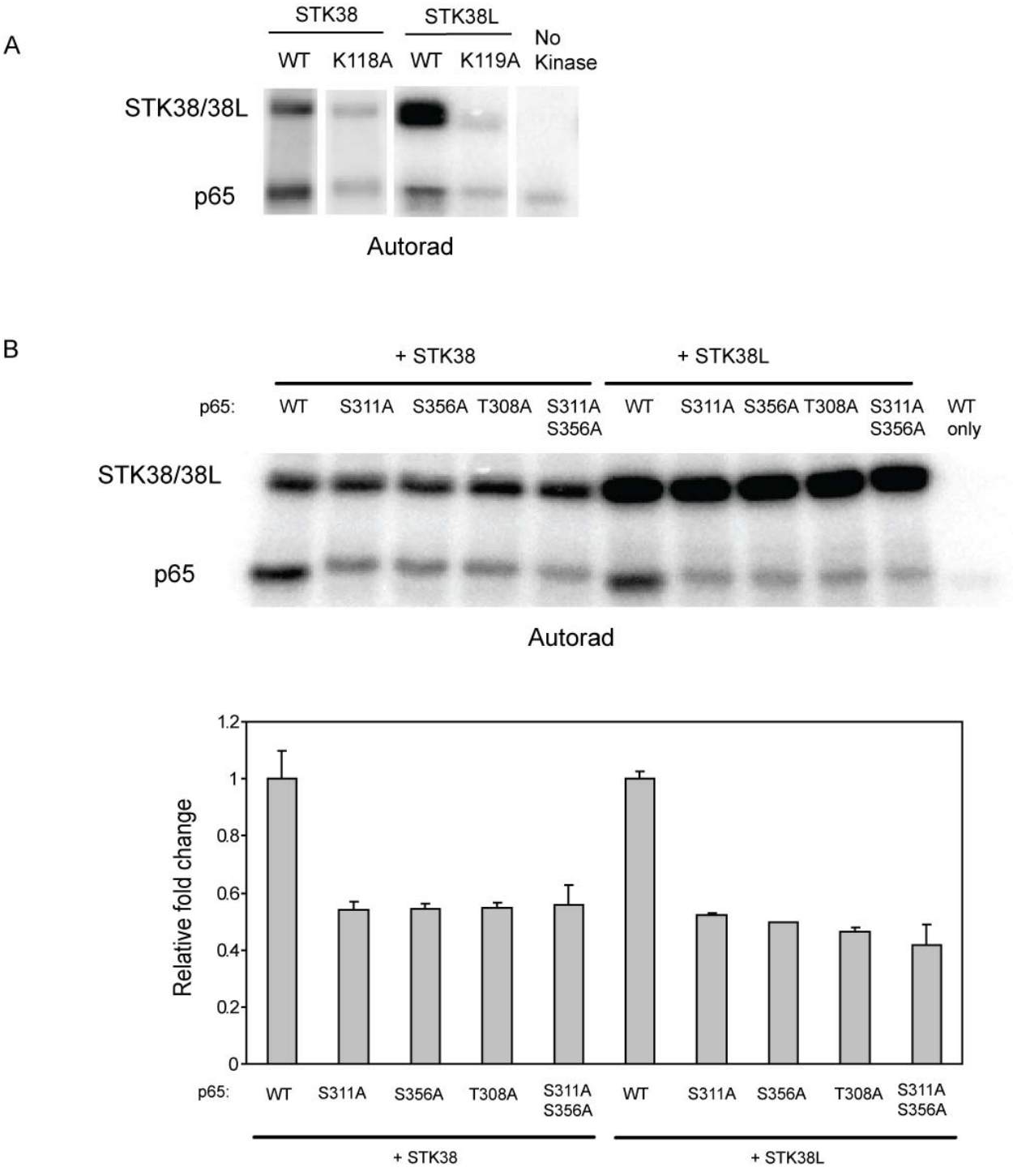

Figure 2.3 In vitro kinase assay. (A) Tagged STK38, STK38L kinases and p65 substrate were expressed in 293T cells. After affinity purification, proteins were run on SDS-PAGE gel and stained with Coomassie blue to estimate their relative levels. In-vitro kinase assays were

performed in which the amount of substrate added was at least twice that of kinase. The STK38 K118A and STK38L K119A represent kinase-dead mutants. All samples were run on the same gel but cut from different lanes. (B) Three p65 phosphorylation sites, T308, S311 and S356, were identified with high certainty by phospho-mass spectrometry of p65 phosphorylated in vitro. p65 mutants were then expressed in cells and kinase assays performed as described in (A); the amounts of different p65 substrates were similar in each reaction. Lower plot quantifies phosphorylation of various substrates, normalized to p65 wild-type. Error bars represent the standard deviation of the mean of biological duplicates.

2.2.3 STK38 affects p65 gene specific regulation

To determine whether phosphorylation of NF-κB by STK38/38L is involved in gene transcription, we created cell lines in which either p65 or the STK38/38L kinases were stably knocked down using RNAi. Because STK38 and STK38L are highly homologous (~87% identical) and have previously been shown to compensate for each other in knockout animals (Cornils et al., 2010; Hergovich et al., 2008), we targeted both proteins in a single cell line. shRNA directed against STK38L was expressed constitutively from a pGIPZ lenti-vector (Sigma) while an inducible shRNA against STK38 derived from a pTRIPZ lenti-vector (Sigma) was expressed under control of a Tet promoter that could be induced by adding doxycycline; shRNA against p65/RELA was independently expressed in a separate cell line under the same inducible Tet promoter. By Western blotting of extracts from shRNA expressing cells, we observed 60-80% reductions in the levels of STK38 and p65 proteins and ~60% reduction in STK38L, depending on the duration and concentration of doxycycline treatment (Figure 2.4A). Using Affymetrix expression microarrays we then measured changes in TNF-α –mediated gene expression following exposure of p65-knockdown or STK38/38L-knockdown cells to 2 μg/ml doxycycline for five days. We reasoned that genes co-regulated by p65 and STK38/38L should display similar changes in expression in the two knockdown lines as compared to control cells expressing non-specific shRNA. By this criterion, genes encoding lymphotoxin beta (LTB) and

tumor necrosis factor alpha (TNF) were selected for further analysis. Both genes are known from previous studies to be upregulated by NF-κB in the presence of TNF-α (Kuprash et al., 1996; Shakhov et al., 1990). A list of genes upregulated by TNF-α stimulation is presented in supplemental Table-S-2.2. Extended lists of genes whose expressions were affected by only p65 knockdown or STK38/38L double knockdown were presented from supplemental Table-S-2.3 to Table-S-2.6.

Real-time PCR assays confirmed that following exposure of cells to 100 ng/ml TNF-α for 6 hours, LTB was induced ~28 ± 4 fold (representing the mean and standard deviation of the mean of biological duplicates) in cells expressing a non-specific control RNA, but only ~6 ± 0.2 fold in cells in expressing shRNA directed against p65 and ~12 ± 2 in cells co-expressing shRNA against STK38/38L (Figure 2.4B). A similar pattern of regulation was observed for the TNF gene (Figure 2.4C). In contrast, full induction of another NF-κB regulated gene, IκBα, was observed to require p65 but not STK38/38L (Figure 2.4D). This implies that STK38/38L kinases are involved in the induction of some but not all NF-κB-regulated genes.

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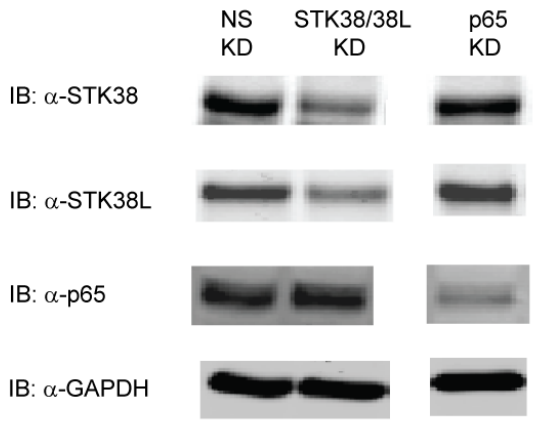
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p65 KD
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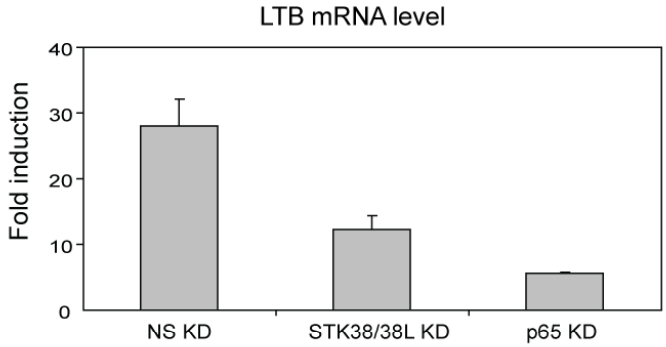
IB: α-p65
IB: α-GAPDH

B


LTB mRNA level
Fold induction
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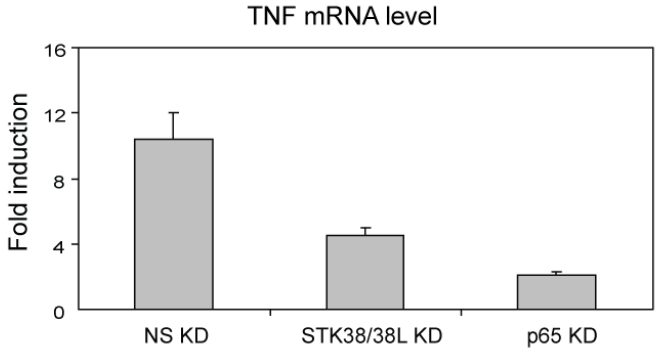
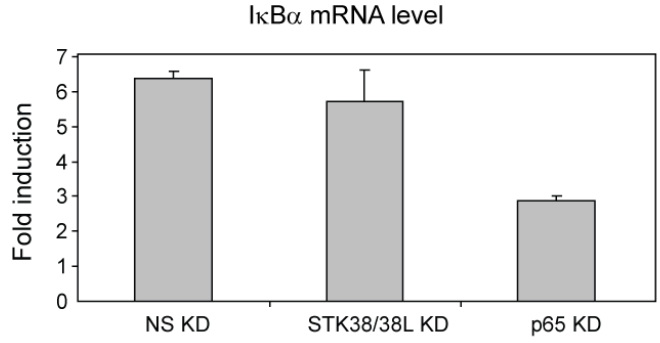
NS KD
STK38/38L KD
p65 KD

C


TNF mRNA level
Fold induction
16
12
8
4
0
NS KD
STK38/38L KD
p65 KD

D


IκBα mRNA level
Fold induction
7
6
5
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NS KD
STK38/38L KD
p65 KD

Figure 2.4 Genes co-regulated by STK38 and p65 in cells treated with 100 ng/mL TNF-α. (A) Western blots of STK38, STK38L and p65 protein in cells expressing non-specific shRNA (NS KD) or shRNA directed against STK38/38L or p65 as indicated. shRNAs were induced by treating cells with 2 μg/ml doxycycline for five days. No significant reduction in p65 protein levels were observed in STK38/38L KD cells as compared to NS KD cells. All samples were run on the same gel but cut from different lanes. (B, C) Real-time PCR was performed to confirm the microarray data. mRNA samples from NS KD, STK38/38L KD and p65 KD cells treated with 100 ng/mL TNF for 0 and 6 hours were collected for qPCR. Fold induction was calculated based on the difference in the number of cycles between the TNF-treated and untreated cells. Error bars represent the standard deviation of the mean of biological duplicates. (D) Expression of IκBα is regulated by p65 but not STK38/38L.

To demonstrate the specificity of shRNA-mediated STK38 knockdown, we carried out rescue experiments in which an RNAi-resistant form of STK38 was transiently expressed in STK38/38L knockdown cells. To avoid artifacts associated with over-expression, cells were examined in which total STK38/38L proteins levels (representing residual endogenous protein expression plus ectopic expression) was at or below that of wild-type cells (Figure 2.5A, B). We observed that expression of RNAi-resistant STK38 in shRNA-producing cells was sufficient to restore capacity to induce LTB up to wild-type levels upon TNF-α ligation (Figure 2.5C); rescue was also observed for TNF-α-mediated induction of the TNF gene (Figure 2.5D). In contrast, re-expression of a kinase-dead STK38-K118A at a similar level did not rescue the induced expression of either gene above the negative control. However, co-immunoprecipitation experiments showed that STK38-K118A bound to p65 to a similar extent as wild-type STK38 (Figure 2.5G), suggesting that kinase-dead STK38-K118A is correctly expressed and folded. Basal expression levels for LTB and TNF mRNA were unaffected by STK38/38L knockdown, but re-expression of either wild-type or kinase-dead STK38/38L increased TNF mRNA levels by ~1.7 fold (Figure 2.5E, F). The reasons for this are unknown but might reflect the sensitivity of the TNF regulatory circuit to transfection conditions. Importantly, changes in basal gene

expression do not affect our conclusions since the data shown in Figure 2.5C and D represent fold-changes between the TNF-treated and untreated cells. We conclude that full induction of LTB and TNF (by exogenous TNF-α) requires both p65 and the kinase activity of STK38/38L. STK38/38L kinase activity is not required for binding to p65 however, suggesting that a downstream process is involved.

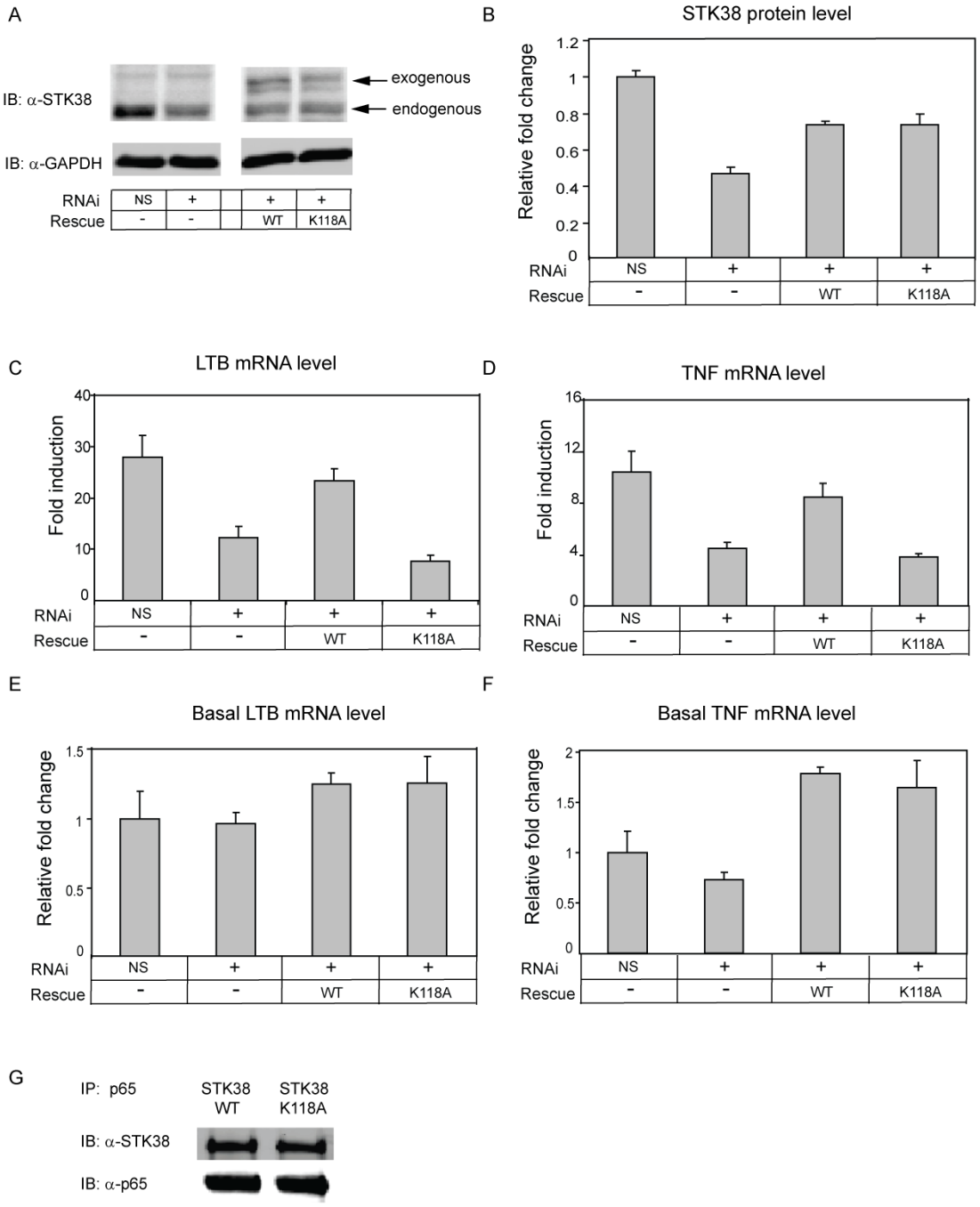

Figure 2.5 shRNA rescue phenotypes. (A) Western blots showing the expression of STK38 in NS KD and STK38/38L KD cells and in cells in which RNAi-resistant forms of either STK38 or STK38 K118A were re-expressed. Re-expressed proteins are larger by ~10 kDa due to the presence of the NTAP tag. All samples were run on the same gel but cut from different lanes. (B) Quantification of rescue level from (A), the level of STK38 in NS KD was normalized to 1. Error bars represent the standard deviation of the mean of biological duplicates. (C, D) Rescue of STK38/38L KD phenotypes in cells by re-expression of either STK38 wild-type (WT) or kinase-dead (K118A) vectors (refer to Materials and Methods for experimental details). Cells were

treated with 100 ng/mL TNF for 6 hours before mRNA isolation and analysis by real-time PCR. Fold induction was calculated based on the difference in the number of cycles between the TNF-treated and untreated cells. (+) denotes STK38/STK38L shRNA. Error bars represent the standard deviation of the mean of biological duplicates. (E, F) mRNA level in various cells populations in the absence of TNFα exposure. In the case of TNF, transfected cells had higher basal gene expression compared to untransfected cells. (G) Pull down of STK38 and STK38 K118A shows that wild type STK38 and kinase dead STK38 K118A bind p65 equally well.

We attempted to increase the extent to which STK38/38L knockdown inhibited LTB and TNF expression by increasing the duration of shRNA expression. When shRNA-expressing cells were exposed continuously to doxycycline for 7 days without changing media, STK38 was present at <10% of wild-type levels and LTB and TNF inducibility by TNF-α was almost completely abolished. However, this defect could not be rescued by re-expression of wild-type STK38, implying that shRNA-mediated STK38 knockdown had lost its selectivity (data not shown). We therefore chose to work with the less severe, but demonstrably selective knockdown phenotypes.

2.2.4 Functional role for p65 residues modified by STK38/38L

To determine whether modification of p65 by STK38/38L plays a role in gene regulation, we re-expressed p65 carrying serine- or threonine-to-alanine point mutations in cells in which endogenous p65 had been knocked down by shRNA (Figure 2.6A, B). In agreement with previous data (Karin and Lin, 2002; Kuprash et al., 1999) we observed that even modest p65 over-expression (to levels ~1.5X that of wild-type) caused activation of NF-κB-responsive genes even in the absence of an exogenous stimulus. This presumably arises because the concentration of endogenous plus ectopic NF-κB overwhelms the ability of IκB to act as an inhibitor. LTB and TNF mRNA levels were therefore measured in p65 knockdown cells re-expressing p65 mutants

in the absence of TNF-α treatment. Data were expressed as the fold-difference in LTB and TNF mRNA levels between cells expressing only p65 shRNA and those expressing both shRNA and RNAi resistant wild-type or mutant p65 proteins. When wild-type p65 was over-expressed, we observed ~13-fold induction of LTB and ~36-fold induction of TNF (Figure 2.6C, D). Expression of the p65 T308A or p65 S311A mutants did not significantly alter the induction of LTB and TNF as compared to wild-type p65, but p65 S356A was roughly half as potent (in all cases we determined that wild-type and mutated p65 proteins were expressed at similar levels as judged by Western blotting and RT-PCR). The effects of the S356A mutation were gene-specific because the levels of the IκBα gene were indistinguishable in the presence of re-expressed wild-type and mutant p65 (Figure 2.6E). Next we asked whether mutating S356 to aspartic acid to mimic the effects of protein phosphorylation would activate p65. We observed that LTB and TNF mRNAs were twice as abundant in cells expressing p65-S356D as compared to wild type p65 when expression levels for the two proteins were similar (Figure 2.6F, G). From these data we conclude that the most important site of p65 modification by STK38/38L is S356 and the phospho-S356 p65 is more active on a subset of NF-κB regulated genes

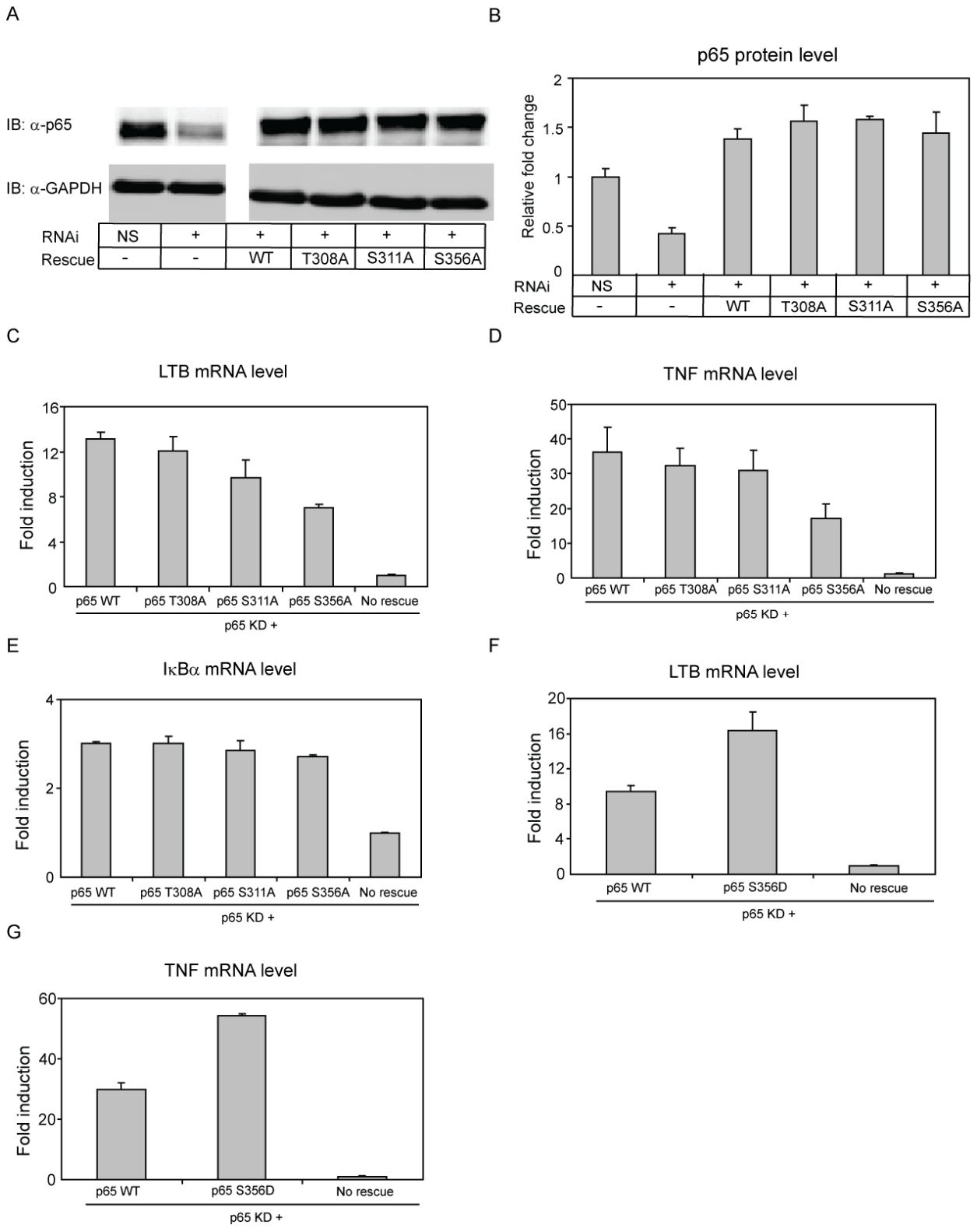

Figure 2.6 Re-expression of wild-type p65 and p65 mutants in p65 knockdown cells. (A) Western blots of p65 and GAPDH levels in NS KD, p65 KD cells and p65 KD cells re-

expressing wild-type p65 or p65 containing various phosphorylation site mutations. (+) denotes p65 KD conditions (expression of p65 shRNA). NS denotes expression of non-specifics shRNA. All samples were run on the same gel but cut from different lanes. (B) Quantification of proteins level in experiments described in (A); the level of p65 in NS KD was normalized to 1. (+) denotes p65 shRNA. Error bars represent the standard deviation of the mean of biological duplicates. (C, D, E, F, G) Phenotypes associated with expression of WT p65 or p65 carrying phosphorylation site mutations (T308A, S311A, S356A and S356D) in p65 KD cells (refer to Materials and Methods for experimental details). Fold induction was calculated based on the difference in the number of real-time PCR cycles between p65 re-expressed and p65 KD cells. Because ectopic p65 expression causes induction of gene expression, TNFα was not added. Error bars represent the standard deviation of the mean of biological duplicates.

2.2.5 Efficient binding of p65 to target genes requires STK38/38L

To determine whether STK38/38L is involved in the binding of p65 to the LTB and TNF genes we performed chromatin immunoprecipitation (ChIP) experiments. Cells were exposed to 1% formaldehyde for 10 minutes to crosslink DNA binding proteins to chromatin, complexes containing p65 were isolated by immunoprecipitation and levels of promoter binding measured by qPCR. For this assay, PCR primers were selected that amplified known κB sequences located within1 kb of the start of transcription for each gene. The amount of p65 binding was quantified as the fold change in qPCR signal between cells treated with or without TNF-α for 90 minutes. In STK38/38L double knockdown cells, we observed a 2-fold reduction in p65 binding to LTB and TNF as compared to control cells, whereas p65 binding to the IκB promoter was not affected (Figure 2.7). We conclude that STK38/38L is required for efficient binding of p65 to the promoters of target genes, a form of regulation that has described for other phosphorylation events on NF-κB subunits (Mosialos and Gilmore, 1993).

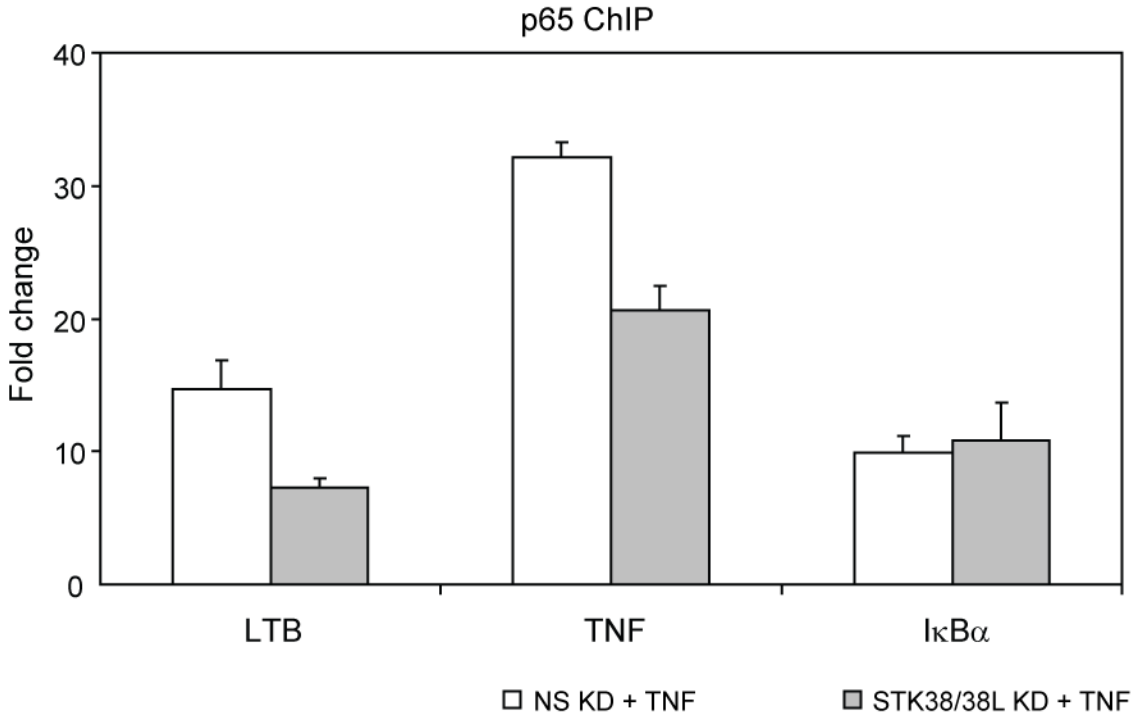

Figure 2.7 Chromatin immunoprecipitation (ChIP) of p65. Binding of p65 to a κB element in the LTB, TNF or IκBα promter in cells treated with exogenous TNF-α. Fold-change in binding was quantified from real-time PCR data and represents the difference in samples from TNF-treated and untreated cells. Error bars represent the standard deviation of four biological replicates.

2.3 DISCUSSION

In this paper we use affinity purification and mass spectrometry to identify proteins that interact with human p65/RELA, one of the most abundant components of the NF-κB transcription factor. We recovered many established p65-binding proteins and also four proteins not previously known to associate with NF-κB. Follow-on co-immunoprecipitation experiments confirmed the interaction (direct or indirect) of the serine/threonine kinases STK38 and its close homologue STK38L with p65. STK38 and STK38L, also known as NDR1 and NDR2, are highly conserved in yeast, worms, flies, mice and humans (Hergovich et al., 2006) where they play a role in a variety of cellular processes, including mitotic exit, cytokinesis, centrosome

duplication, cell proliferation and apoptosis (Hergovich et al., 2008). We find that shRNA-mediated knockdown of both STK38 and STK38L (whose activities could not be distinguished) reduces NF-κB –mediated transactivation of TNF-α-responsive genes such as LTB and TNF as well as binding of p65 to LTB and TNF promoters. The product of the TNF gene is the TNF-α cytokine itself, which is well known to be regulated in an autocrine manner and involved in many important cellular processes (Balkwill, 2006). LTB is a membrane-bound receptor whose over-expression causes hepatitis and hepatocellular carcinoma in mice (Balkwill, 2006; Haybaeck et al., 2009). STK38/38L kinases are likely to regulate transcription factors other than p65: microarray analysis shows that additional genes are affected by STK38/38L double knockdown but not by p65 knockdown. This is consistent with a suggestion from Wang et al (Wang et al., 2009) that STK38 is a pleiotropic transcriptional regulator in B cells. However, not all p65-regulated genes are regulated by STK38/38L. IκBα, for example, is activated in a p65-dependent manner by TNFα but it is not affected by STK38/38L knockdown.

Not much is known about the substrates of STK38 and STK38L in mammalian cells. We find that p65 is an STK38/38L substrate in vitro and that phosphorylation occurs on residues T308, S311 and S356. Substituting alanine at any one of these p65 residues reduces phosphorylation by STK38/38L by ~50%, but double mutants (e.g. p65-S311A/ S356A) have similar effects to single mutants. This implies a pattern of multi-site phosphorylation in which mutation of preferred phosphorylation sites causes other residues become modified, a fairly common phenomenon with kinases that bind avidly to their substrates (e.g. (Jeffery et al., 2001)).

Our ability to dissect STK38/38L phenotypes is negatively impacted by challenges of retaining selectivity in shRNA knockdown phenotypes of pro-inflammatory signaling molecules. We have explored a wide range of knockdown conditions, testing in each case whether observed

phenotypes could be rescued by ectopic expression of an RNAi-resistant wild-type gene. We observe that as STK38/38L knockdown phenotypes become stronger (with greater duration of shRNA expression) rescue becomes less effective, suggesting a problem with RNAi specificity. Anecdotally we have found this problem to be more severe with STK38/38L than with unrelated regulatory processes, perhaps reflecting strong feedback regulation in the NF-κB pathway. From a practical perspective, further analysis is likely to require cells in which STK38/38L activity can be inactivated by a means other than shRNA. Creating knock-in cells that expressed active-site STK38/38L mutants that can be acutely inhibited by 1-NAPP1 (using methods developed by Shokat and colleagues (Blethrow et al., 2004)), would be one way forward.

Despite the experimental challenges, four pieces of data argue that STK38/38L is a NF-κB regulator: (i) STK38/38L binds to p65 and phosphorylates it in vitro (ii) STK38/38L knockdown cells are less efficient at transactivation of TNF and LTB genes and this defect is not rescued by kinase-dead STK38/38L variants (iii) phosphorylation of p65 at S356 site is necessary for full LTB and TNF induction and the kinase activity of STK38/38L is required for efficient binding of p65 to promoters (iv) a phospho-mimetic alanine to aspartate mutation, p65-S356D, increases transcription of LTB and TNF above wild type levels. A model of STK38's role in p65-dependent inflammatory gene activation is proposed in Figure 2.8.

Despite above evidence, we have not conclusively proven that STK38/38L modification of p65 is the key regulatory event in vivo. p65 is known to be modified by many kinases (modification of S311 by PKCζ activates p65 for example (Duran et al., 2003)) and given this complexity it is difficult to establish unambiguous cause-effect relationships between specific kinases and transcriptional activity. However, no other kinase has previously been reported to modify p65-S356 nor has the residue been shown to play a role in NF-κB transcription. Thus,

we believe the most likely hypothesis to be that STK38/38L are p65 co-factors that function by modifying p65-S356 and increasing DNA binding activity.

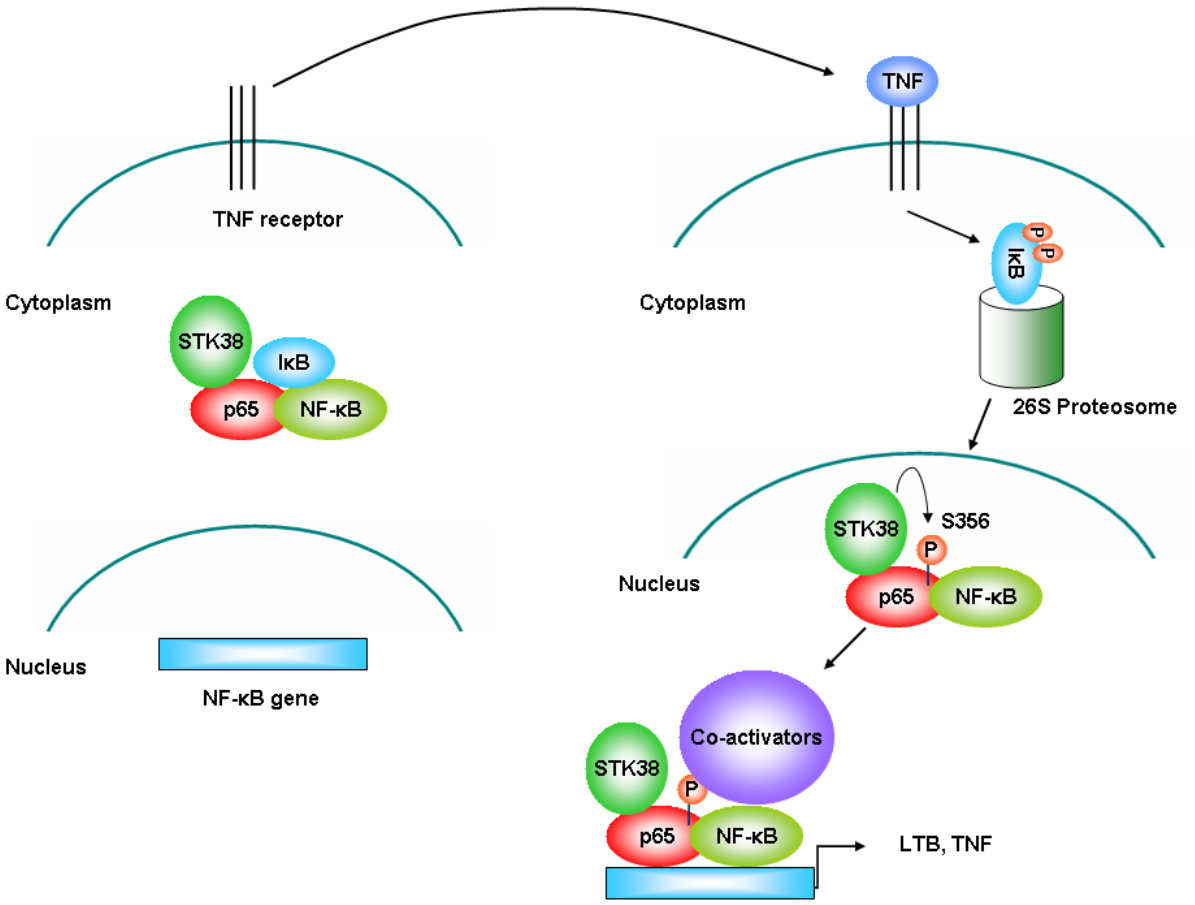

Figure 2.8 A proposed model of STK38's role in p65-dependent inflammatory gene activation. In unstimulated conditions, STK38 binds an NF-κB complex containing p65. This complex is sequestered in the cytoplasm due to its association with an IκB or IκB-equivalent protein. Upon TNF stimulation, IκB is degraded by the 26S proteosome, which allows NF-κB to enter the nucleus. Also, with the degradation of IκB, serine 356 of p65 is phosphorylated by STK38, which facilitates recruitment of transcriptional co-activators at selected NF-κB gene promoters and resulting in enhancement of gene transcription.

2.4 MATERIALS AND METHODS

Cell lines and Reagents

Human colorectal carcinoma HCT116 cells were cultured in McCoy5A media plus 10% FBS and 1% penicillin/streptomycin. HEK 293T cells were cultured in DMEM with the same supplements as HCT116. The following antibodies were used: anti-p65 (sc-109, sc-372 from Santa-Cruz); anti-GAPDH (from Abcam); anti-STK38 (from Abnova); anti-STK38L (gift from Dr. Hemmings, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland); anti-Streptag II (from Qiagen). Other reagents were purchased from the following vendors: TNF-α from PeproTech; SYBR reaction mix from Applied Biosystems; TurboFect in vitro transfection reagent from Fermentas; anti-FLAG and anti-HA resin from Sigma., Production of adeno-associated viruses and screening for resistant clones carrying the knock-in construct were done according to protocol from the Vogelstein and Bunz laboratories (Rago et al., 2007). Once, a knock-in clone was identified, the selection cassette was removed using Ad-Cre (adeno viruses expressing Cre-recombinase) to ensure proper expression of the knock-in protein.

Plasmid Constructs

The AAV p65 knock-in construct was generated by fusing the left homology arm (LHA), SEPT fragment containing a neo selection gene (from pSEPT vector, gift from the Bunz laboratory, Johns Hopkins University School of Medicine, Maryland, USA) and the right homology arm (RHA) by PCR. The LHA and RHA are about 1kb in length and contain the NotI restriction sites at the outer left of the LHA and outer right of the RHA. The fusion PCR product was cloned into the NotI sites of pAAV-MCS. Plasmids expressing candidate p65 interactors were constructed using the following restriction sites: BamHI and SalI for STK38, CCT6A, PRMT1, SERBP1; BamHI and EcoRI for STK38L. Fragments were cloned into the pNTAP-C

vector (Stratagene) containing the tandem affinity Streptavidin and Calmodulin-binding peptide tag at the N terminus. Also, a StrepTag II sequence (Qiagen) was included for Western blot detection. After being cloned into the pNTAP-C vector, the following genes, STK38 (cut with BglII and SacII), STK38L (cut with EcoRI and SacII), CCT6A (cut with BglII and SacII), SERBP1 (cut with NheI and EcoRI) were inserted into pEGFP-N1 vector. This serves two purposes, the first for separating them from the p65 band on Western blots and the second for visualization of protein expression. The p65 expression vector was made by adding an HA tag sequence at the beginning of the gene and inserting it in the pEGFP-N1 with p65's stop codon included. All cDNAs for cloning were generated by RT-PCR from the mRNA extract of HCT116 cells. Point mutations introduced into STK38 (K118A), STK38L (K119A), p65 (T308A, S311A, S356A, S311A S356A, S356D) were created using the site-directed mutagensis kit from Strategene. pAAV-MCS, pHelper and pRC vectors were gifts from the Lois laboratory, Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, USA.

Liquid chromatography/Mass Spectrometry (LC/MS) Analysis

Each MS sample was prepared from $5x10^8$ cells. Cells were lysed using lysate buffer (0.1% NP-40 in 25 mM HEPES pH 7.5, 10% glycerol supplemented with protease inhibitors leupeptin, pepstatin, chymostatin and phosphatase inhibitors sodium orthovanadate, sodium fluoride, and beta-glycerol phosphate). Affinity purification was carried out using anti-FLAG resin. Bound protein complexes were eluted using 0.1 M glycine HCl, pH 3.5. Proteins were precipitated by adding TCA (6%) and sodium deoxycholate (0.02%), samples were placed on ice for 1 hour then centrifuged, pellets were washed with ice-cold acetone and resuspended in 10% acetonitrile in 50 mM sodium bicarbonate solution. Four different samples representing parental

cells treated with and without 100 ng/mL TNF-α and knocked-in p65-tagged HCT116 cells with and without TNF-α were submitted for MS analysis on the LTQ-FT machine from Thermo Electron. For phospho-MS analysis of p65 sites, an in vitro kinase was carried out using ATP followed by SDS-PAGE. The gel was stained with Colloidal Blue (Invitrogen) and the band corresponding to p65 was excised and submitted for phospho-mass spectrometry using the LTQ-FT machine from Thermo Electron. To maximize the number of phospho peptides identified, each sample was digested with either trypsin alone or trypsin/chymotrypsin together. All mass spectrometry analyses were performed in the Taplin mass spectrometry facility at the Harvard Medical School.

Reciprocal immunoprecipitation

Candidate proteins and p65 were expressed separately in HEK 293T cells by transient transfection. 24-hour post-transfection, cell lysates were collected. To measure the binding of each candidate protein to p65, lysates from cells expressing a tagged version of each protein were mixed with lysate from HEK 293T cells over-expressing p65, or in the case of data shown in Figure 2.2C and D with lysates from HCT116 cells expressing p65 at endogenous levels. Mixtures were rotated overnight at 4^{o}C with Strep-Tactin Superflow Plus (Qiagen) resin to pull down the tagged proteins followed by analysis on SDS-PAGE gels; anti-p65 antibodies were used to measure the extent of binding to p65 by Western blotting.

Gene expression profiling and data analysis

mRNA samples were prepared by using the NucleoSpin RNA II kit (Macherey-Nagel). cDNA was synthesized and labeled before hybridizing on the Affymatrix Human U133A 2.0 chips according to manufacturer's protocol. Biological duplicates were prepared for each condition. Expression data were normalized using quantile normalization. Genes were

considered to be affected if either p65 or STK38/38L knockdown cells exhibited decreased expression by >1.8-fold compared to non-specific knockdown cells when treated with 100 ng/mL TNF-α for 6 hours. To select for genes whose expressions were dependent on TNF-α treatment, a >1.8-fold increase cut-off was chosen when comparing expression data between untreated and treated samples. Microarray data collection was performed at the BioMicro Center at the Massachusetts Institute of Technology.

Quantitative real-time PCR and Chromatin Immunopreciptation Assays

mRNA samples were extracted from cells using the NucleoSpin RNA II kit (Macherey-Nagel). cDNA synthesis was done using RETROscript kit with oligo (dT) (Applied Biosystems). qPCR reactions were performed in duplicate by using SYBR Green PCR Master Mix (Applied Biosystems) in the Eppendorf Realplex Mastercycler S machine. Primers are listed in supplementary Table S-2.7. The relative transcription level was calculated by using the ΔΔCt method with GAPDH as a normalization control.

For Chromatin Immunopreciptation (ChIP) assays, cells were treated with TNF-α for 90 minutes followed by fixation with 10% formaldehyde (1% final concentration) for 10 minutes. Cells were lysed first in swelling buffer for 15 minutes (25mM HEPES pH 7.8, 1.5 mM $MgCl_2$, 10mM KCl, 0.1% NP-40, 1mM DTT, protease inhibitors (leupeptin, pepstatin, chymostatin) then in sonication buffer (50mM HEPES pH 7.8, 140mM NaCl, 1mM EDTA, 0.1% SDS, 1% TritonX-100 supplemented with protease inhibitors (leupeptin, pepstatin, chymostatin). Samples were sonicated for 3 minutes with 20-second pulse intervals and 1 minute off at each interval at 4^oC using a Fisher-Scientific 550 Sonic Dismembrator with output setting at 3.5. Chromatin was immunoprecipitated using an anti-p65 (sc-372 Ab from Santa-Cruz) and Dynabead Protein A

(Invitrogen) at 4°C overnight. DNA was purified using phenol/chloroform precipitation method. Real-time PCR was used to quantify promoter's binding.

In vitro kinase assays

Both kinases and substrates were generated by transient transfection of tagged proteins in HEK 293T cells using TurboFect in vitro transfection reagent (Fermentas). Kinases were purified using Strep-Tactin Superflow Plus resin (eluted with d-desthiobiotin; Sigma) and substrates were purified using anti-HA resin (eluted with HA peptide; Sigma). Kinases were first incubated with 1x kinase buffer (25 mM HEPES pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$, 2 μM unlabelled ATP) for 15 minutes at room temperature to allow for their activation. Substrates were then added together with 5 μCi of [γ-32P]ATP in a 50 μl reaction volume. Reactions were stopped using SDS sample buffer after 50 minutes at room temperature. Proteins were resolved by 8% SDS–PAGE, gels were dried and ^{32}P incorporation was measured by autoradiography.

shRNA knockdown cells

Doxycyclin (Dox)-inducible shRNA lentivirus-based vectors for STK38, p65 and non specific (NS) targets and non-inducible vectors for STK38L were purchased from Open Biosystems. Viruses were made using HEK 293T according to manufacturer's protocol using helper plasmids pCGgag/pol, and pVSV-G (gifts from the Elledge laboratory, Harvard Medical School, Boston, USA). Knock-in HCT116 cells were infected using 4-8 μg/ml polybrene. Viruses were removed after 24 h. Cells were then selected with puromycin (0.5-2μg/ml) to remove uninfected population. To make STK38/STK38L double knockdown cells, STK38 single knockdown cells were generated first and these cells were used for a second round of infection with viruses carrying the STK38L shRNA vector. Since the shRNA vector for STK38L

expresses a GFP reporter, sorting was used to select for cells that were successfully infected with the STK38L shRNA vector.

To determine if phenotypes associated with shRNA expression could be rescued by expression of wild-type or mutated genes, shRNA-expressing cells were treated with Dox for 72 hours to knock down target genes. The knockdown genes were rescued by transiently transfecting vectors carrying these genes. The shRNA against STK38 is toward the 3'UTR, so rescuing with STK38 cDNAs require no further modifications. However, for p65 rescue, silent mutations were introduced into the region of plasmids carrying p65 cDNAs where p65's shRNA recognizes by site directed mutagenesis. Approximately, four hours post-transfection, media was changed to a 2% serum media plus Dox. Cells were allowed to grow for four more days prior to TNF-α stimulation (STK38 rescue) followed by mRNA extractions and real-time PCR.

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2.6 SUPPLEMENTAL INFORMATION

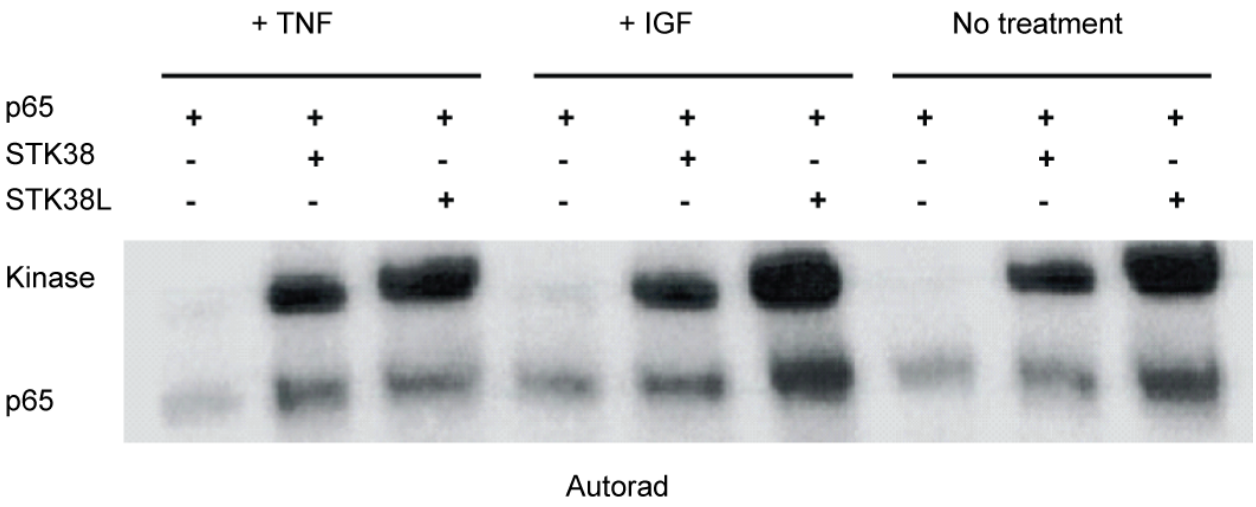


Figure S-2.1 In vitro kinase assay. Kinases and p65 substrate were expressed in 293T cells. Cells expressing kinases were treated with 100 ng/mL TNF-α or IGF for 6 hours before lysates were collected. After affinity purification, proteins were run on SDS-PAGE gel and stained with Coomassie blue to estimate their relative levels. In-vitro kinase assay was performed in which the amount of substrate added was at least twice that of kinase. Purified STK38 and STK38L from untreated or TNF-α or IGF treated cells can phosphorylate p65 and there appears to be no significant difference in p65's phosphorylation signal between these conditions.

Table-S-2.1 p65 binding partners. Lysate samples from HCT116 cells carrying a p65 expressing a FLAG tag were run through an anti-FLAG resin column. Eluted fractions containing p65 interacting proteins were analysed by mass spectrometry. Candidate p65 interactors were selected if they were reproducibly identified in two independent experiments and were represented by at least two different peptides.

Gene symbol	Gene name
ANM5	Protein arginine n-methyltransferase 5
HSP7C	Heat shock cognate 71 kda protein
GRP78	78 kda glucose-regulated protein precursor
TF65	Nuclear factor nf-kappa-b p65 subunit
GRP75	75 kda glucose-regulated protein
STK38	Serine/threonine-protein kinase 38
ST38L	Serine/threonine-protein kinase 38-like
NFKB1	Nuclear factor nf-kappa-b p105 subunit
ATPB	ATP synthase subunit beta
HS70L	Heat shock 70 kda protein 1-like
ANM1	Protein arginine n-methyltransferase 1
HS90A	Heat shock protein hsp 90-alpha
TBA2	Tubulin alpha-2 chain
RSSA	40s ribosomal protein sa
IKBE	NF-kappaB inhibitor epsilon
HSP72	Heat shock-related 70 kda protein 2
ANXA1	Annexin a1
G3P	Glyceraldehyde-3-phosphate dehydrogenase
EZRI	Ezrin
HSP71	Heat shock 70 kda protein 1
PAIRB	Serpine1 mrna-binding protein 1
CDK9	Cell division protein kinase 9
NFKB2	Nuclear factor nf-kappa-b p100 subunit
TBB1	Tubulin beta-1 chain
ATPA	ATP synthase subunit alpha
IKBA	NF-kappaB inhibitor alpha

REL	c-Rel protein
IKBB	NF-kappaB inhibitor beta

Table-S-2.2 Genes upregulated by TNF-α stimulation. NS KD HCT116 cells were treated with 100 ng/mL TNF-α or mock control for 6 hours before mRNA samples were collected. cDNA was synthesized and labeled before hybridizing on the Affymatrix Human U133A 2.0 chips according to manufacturer's protocol. Biological duplicates were prepared for each condition. GC-RMA method was used to extract the gene expression values. Expression data were then normalized using quantile normalization. To select for genes whose expressions were dependent on TNF-α treatment, a >1.8-fold increase cut-off was chosen when comparing expression data between untreated and treated samples.

Gene symbol	Gene name
BIRC3	baculoviral IAP repeat-containing 3
IL8	interleukin 8
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
APOL6	apolipoprotein L, 6
APOL3	apolipoprotein L, 3
UBD	ubiquitin D
TNFAIP3	tumor necrosis factor, alpha-induced protein 3
TNFRSF9	tumor necrosis factor receptor superfamily, member 9
PSMB9	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)
CXCL2	chemokine (C-X-C motif) ligand 2
IFI44	interferon-induced protein 44
TNFAIP2	tumor necrosis factor, alpha-induced protein 2
SAA1, SAA2	serum amyloid A1, serum amyloid A2
ICAM1	intercellular adhesion molecule 1
IFIH1	interferon induced with helicase C domain 1
TLR3	toll-like receptor 3
CCL20	chemokine (C-C motif) ligand 20
UBE2L6	ubiquitin-conjugating enzyme E2L 6
TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)
ISG20	interferon stimulated exonuclease gene 20kDa
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
CD83	CD83 molecule
IFIT3	interferon-induced protein with tetratricopeptide repeats 3
LTB	lymphotoxin beta (TNF superfamily, member 3)
STAT1	signal transducer and activator of transcription 1, 91kDa
NMI	N-myc (and STAT) interactor
TYMP	thymidine phosphorylase
IRF1	interferon regulatory factor 1
IL15	interleukin 15
LAMP3	lysosomal-associated membrane protein 3
DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60
OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa
BTN3A1	butyrophilin, subfamily 3, member A1
IFI35	interferon-induced protein 35
ERAP1	endoplasmic reticulum aminopeptidase 1

HCP5	HLA complex P5
SAMD9	sterile alpha motif domain containing 9
IFI6	interferon, alpha-inducible protein 6
GBP1	guanylate binding protein 1, interferon-inducible, 67kDa
USP18	ubiquitin specific peptidase 18
OPTN	optineurin
TNIP1	TNFAIP3 interacting protein 1
ZC3HAV1	zinc finger CCCH-type, antiviral 1
BST2	bone marrow stromal cell antigen 2
NAV3	neuron navigator 3
SERPINB1	serpin peptidase inhibitor, clade B (ovalbumin), member 1
B2M	beta-2-microglobulin
APOL2	apolipoprotein L, 2
CYLD	cylindromatosis (turban tumor syndrome)
CASP4	caspase 4, apoptosis-related cysteine peptidase
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)
LRRC49	leucine rich repeat containing 49
MUC1	mucin 1, cell surface associated
SP110	SP110 nuclear body protein
RASGRP3	RAS guanyl releasing protein 3 (calcium and DAG-regulated)
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58
SERPINB9	serpin peptidase inhibitor, clade B (ovalbumin), member 9
CXCL11	chemokine (C-X-C motif) ligand 11
ISG15	ISG15 ubiquitin-like modifier
SECTM1	secreted and transmembrane 1
NAV2	neuron navigator 2
PPARD	peroxisome proliferator-activated receptor delta
PLAU	plasminogen activator, urokinase
HDAC9	histone deacetylase 9
APOL1	apolipoprotein L, 1
SP100	SP100 nuclear antigen
HLA-F	major histocompatibility complex, class I, F
SYNGR3	synaptogyrin 3
IFIT5	interferon-induced protein with tetratricopeptide repeats 5
RARRES3	retinoic acid receptor responder (tazarotene induced) 3
CXCL3	chemokine (C-X-C motif) ligand 3
ZBED2	zinc finger, BED-type containing 2
DAPP1	dual adaptor of phosphotyrosine and 3-phosphoinositides
BTN3A3	butyrophilin, subfamily 3, member A3
JUN	jun oncogene
CD40	CD40 molecule, TNF receptor superfamily member 5
IFIT2	interferon-induced protein with tetratricopeptide repeats 2
NFE2L3	nuclear factor (erythroid-derived 2)-like 3
BTN2A2	butyrophilin, subfamily 2, member A2
ETV7	ets variant 7
IFIT1	interferon-induced protein with tetratricopeptide repeats 1
SLC12A7	solute carrier family 12 (potassium/chloride transporters), member 7
CFLAR	CASP8 and FADD-like apoptosis regulator

CCL5	chemokine (C-C motif) ligand 5
SRCAP	Snf2-related CREBBP activator protein
HLA-B	major histocompatibility complex, class I, B
DRAM	damage-regulated autophagy modulator
EHD1	EH-domain containing 1
FAS	Fas (TNF receptor superfamily, member 6)
ITGB8	integrin, beta 8
IFNGR2	interferon gamma receptor 2 (interferon gamma transducer 1)
BCL3	B-cell CLL/lymphoma 3
TAPBP	TAP binding protein (tapasin)
HIVEP1	human immunodeficiency virus type I enhancer binding protein 1
ACSL5	acyl-CoA synthetase long-chain family member 5
LHFP	lipoma HMGIC fusion partner
ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide
SAMD4A	sterile alpha motif domain containing 4A
PLCB4	phospholipase C, beta 4
HIVEP2	human immunodeficiency virus type I enhancer binding protein 2
ATP2C1	ATPase, Ca++ transporting, type 2C, member 1
CLIC5	chloride intracellular channel 5
TAP2	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)
ATF3	activating transcription factor 3
CXCR4	chemokine (C-X-C motif) receptor 4
IL15RA	interleukin 15 receptor, alpha
CLEC2B	C-type lectin domain family 2, member B
BIRC2	baculoviral IAP repeat-containing 2
SOD2	superoxide dismutase 2, mitochondrial
HLA-E	major histocompatibility complex, class I, E
BTG1	B-cell translocation gene 1, anti-proliferative
RC3H2	ring finger and CCCH-type zinc finger domains 2
NRIP1	nuclear receptor interacting protein 1
MALT1	mucosa associated lymphoid tissue lymphoma translocation gene 1
ZNF695	zinc finger protein 695
TRIM21	tripartite motif-containing 21
SEC23B	Sec23 homolog B (S. cerevisiae)
EFNA1	ephrin-A1
BTN3A2	butyrophilin, subfamily 3, member A2
CES2	carboxylesterase 2 (intestine, liver)
PLSCR1	phospholipid scramblase 1
BCL6	B-cell CLL/lymphoma 6
GBP2	guanylate binding protein 2, interferon-inducible
NRG1	neuregulin 1
PTGES	prostaglandin E synthase
DUSP10	dual specificity phosphatase 10
ZC3H7B	zinc finger CCCH-type containing 7B
OASL	2'-5'-oligoadenylate synthetase-like
MUC3B	mucin 3B, cell surface associated
IRF9	interferon regulatory factor 9
ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)

WWC1	WW and C2 domain containing 1
HLA-C	major histocompatibility complex, class I, C
PDE4DIP	phosphodiesterase 4D interacting protein
IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)
PLAUR	plasminogen activator, urokinase receptor
PSME2	proteasome (prosome, macropain) activator subunit 2 (PA28 beta)
MMD	monocyte to macrophage differentiation-associated
OBFC2A	oligonucleotide/oligosaccharide-binding fold containing 2A
PISD	phosphatidylserine decarboxylase
DNPEP	aspartyl aminopeptidase
ZNF160	zinc finger protein 160
CD47	CD47 molecule
PARP8	poly (ADP-ribose) polymerase family, member 8
CD44	CD44 molecule (Indian blood group)
JUNB	jun B proto-oncogene
RELB	v-rel reticuloendotheliosis viral oncogene homolog B
REL	v-rel reticuloendotheliosis viral oncogene homolog (avian)
SLC35E1	solute carrier family 35, member E1
SDC4	syndecan 4
FGD6	FYVE, RhoGEF and PH domain containing 6
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2
RPS17L4	ribosomal protein S17-like 4
DGKE	diacylglycerol kinase, epsilon 64kDa
ELL2	elongation factor, RNA polymerase II, 2
PHF11	PHD finger protein 11
ASMTL	acetylserotonin O-methyltransferase-like
PLK2	polo-like kinase 2 (Drosophila)
IRS2	insulin receptor substrate 2
NBN	nibrin
IFNAR2	interferon (alpha, beta and omega) receptor 2
RAB7A	RAB7A, member RAS oncogene family
IDS	iduronate 2-sulfatase
KTN1	kinectin 1 (kinesin receptor)
IRF2	interferon regulatory factor 2
C3	complement component 3
HSPG2	heparan sulfate proteoglycan 2
STYK1	serine/threonine/tyrosine kinase 1
LIPG	lipase, endothelial
ERAP2	endoplasmic reticulum aminopeptidase 2
MYD88	myeloid differentiation primary response gene (88)
ITM2B	integral membrane protein 2B
ZFP36L1	zinc finger protein 36, C3H type-like 1
ITPKC	inositol 1,4,5-trisphosphate 3-kinase C
OTUD4	OTU domain containing 4
CHMP5	chromatin modifying protein 5
CYR61	cysteine-rich, angiogenic inducer, 61
TANK	TRAF family member-associated NFKB activator
VAMP1	vesicle-associated membrane protein 1 (synaptobrevin 1)

KLF9	Kruppel-like factor 9
SAT1	spermidine/spermine N1-acetyltransferase 1
SAMHD1	SAM domain and HD domain 1
SEMA3C	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C
SMG1	SMG1 homolog, phosphatidylinositol 3-kinase-related kinase (C. elegans)
TRAF3	TNF receptor-associated factor 3
AGPAT4	1-acylglycerol-3-phosphate O-acyltransferase 4 (lysophosphatidic acid acyltransferase, delta)
DSE	dermatan sulfate epimerase
HGSNAT	heparan-alpha-glucosaminide N-acetyltransferase
GRAMD3	GRAM domain containing 3
GALNT6	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 6
MAOA	monoamine oxidase A
ATP8B1	ATPase, class I, type 8B, member 1
FGF2	fibroblast growth factor 2 (basic)
ELK3	ELK3, ETS-domain protein (SRF accessory protein 2)
ZNF136	zinc finger protein 136
GCH1	GTP cyclohydrolase 1
APOBEC3B	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B
SMAD3	SMAD family member 3
PDE4C	phosphodiesterase 4C, cAMP-specific (phosphodiesterase E1 dunce homolog, Drosophila)
PICALM	phosphatidylinositol binding clathrin assembly protein
HEG1	HEG homolog 1 (zebrafish)
TICAM1	toll-like receptor adaptor molecule 1
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
SLC25A22	solute carrier family 25 (mitochondrial carrier: glutamate), member 22
UGCG	UDP-glucose ceramide glucosyltransferase
UBE2H	ubiquitin-conjugating enzyme E2H (UBC8 homolog, yeast)
TRIM6-TRIM34	tripartite motif-containing 34 - tripartite motif-containing 6
PSMB10	proteasome (prosome, macropain) subunit, beta type, 10
EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3
BAZ1A	bromodomain adjacent to zinc finger domain, 1A
STC2	stanniocalcin 2
YOD1	YOD1 OTU deubiquinating enzyme 1 homolog (S. cerevisiae)
MICB	MHC class I polypeptide-related sequence B
RPL35A	Ribosomal protein L35a
PARP12	poly (ADP-ribose) polymerase family, member 12
NKX3-1	NK3 homeobox 1
IL4R	interleukin 4 receptor
MSN	moesin
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)
SGMS1	sphingomyelin synthase 1
GLS	glutaminase
PSMB2	proteasome (prosome, macropain) subunit, beta type, 2
LRCH3	leucine-rich repeats and calponin homology (CH) domain containing 3
MFHAS1	malignant fibrous histiocytoma amplified sequence 1
JMJD3	jumonji domain containing 3, histone lysine demethylase
CASP8	caspase 8, apoptosis-related cysteine peptidase
COPA	coatomer protein complex, subunit alpha

TNFAIP1	tumor necrosis factor, alpha-induced protein 1 (endothelial)
SIPA1L3	signal-induced proliferation-associated 1 like 3
TOR1AIP1	torsin A interacting protein 1
ABTB2	ankyrin repeat and BTB (POZ) domain containing 2
IGSF3	immunoglobulin superfamily, member 3
TDRD7	tudor domain containing 7
ABI1	abl-interactor 1
SPATA2L	spermatogenesis associated 2-like
FBXO38	F-box protein 38
CCRN4L	CCR4 carbon catabolite repression 4-like (S. cerevisiae)
ROD1	ROD1 regulator of differentiation 1 (S. pombe)
RAB27A	RAB27A, member RAS oncogene family
TNF	Tumor necrosis factor alpha

Table-S-2.3 Genes downregulated by p65 knockdown. NS KD and p65 KD HCT116 cells were treated with 100 ng/mL TNF-α for 6 hours before mRNA samples were collected. cDNA was synthesized and labeled before hybridizing on the Affymatrix Human U133A 2.0 chips according to manufacturer's protocol. Biological duplicates were prepared for each condition. GC-RMA method was used to extract the gene expression values. Expression data were then normalized using quantile normalization. To select for genes whose expressions were downregulated by p65 knockdown, a >1.8-fold decrease cut-off was chosen when comparing expression data between ND KD and p65 KD samples.

Gene symbol	Gene name
PLCB4	phospholipase C beta 4
TNFAIP3	tumor necrosis factor alpha-induced protein 3
IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells kinase beta
PTGES	prostaglandin E synthase
BIRC3	baculoviral IAP repeat-containing 3
SPG11	spastic paraplegia 11 (autosomal recessive)
TYMP	thymidine phosphorylase
BTN3A1	butyrophilin subfamily 3 member A1
LMO2	LIM domain only 2 (rhombotin-like 1)
CIR	CBF1 interacting corepressor
HERC5	hect domain and RLD 5
CCNG2	cyclin G2
SERPINB9	serpin peptidase inhibitor clade B (ovalbumin) member 9
ABTB2	ankyrin repeat and BTB (POZ) domain containing 2
IFNGR1	interferon gamma receptor 1
TRIM38	tripartite motif-containing 38
EFNA1	ephrin-A1
CD83	CD83 molecule
IFI6	interferon alpha-inducible protein 6
IFIH1	interferon induced with helicase C domain 1
BTN2A2	butyrophilin subfamily 2 member A2
TNFAIP2	tumor necrosis factor alpha-induced protein 2
CXCL3	chemokine (C-X-C motif) ligand 3
ICAM1	intercellular adhesion molecule 1 (CD54) human rhinovirus receptor
ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6
ITGB8	integrin beta 8
S100PBP	S100P binding protein
TPPP	tubulin polymerization promoting protein
SLC25A15	solute carrier family 25 (mitochondrial carrier; ornithine transporter) member 15
IFNAR2	interferon (alpha beta and omega) receptor 2
LIMK2	LIM domain kinase 2
IL17RB	interleukin 17 receptor B
DGKE	diacylglycerol kinase epsilon 64kDa
ADD3	adducin 3 (gamma)
MAP3K5	mitogen-activated protein kinase kinase kinase 5

IFNGR1	interferon gamma receptor 1
OS9	amplified in osteosarcoma
ALCAM	activated leukocyte cell adhesion molecule
PCDH1	protocadherin 1
SOD2	superoxide dismutase 2 mitochondrial
PLCB4	phospholipase C beta 4
EGFR	epidermal growth factor receptor
SP110	SP110 nuclear body protein
TNF	tumor necrosis factor alpha
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha

Table-S-2.4 Genes downregulated by STK38/38L knockdown. NS KD and STK38/38L KD HCT116 cells were treated with 100 ng/mL TNF-α for 6 hours before mRNA samples were collected. cDNA was synthesized and labeled before hybridizing on the Affymatrix Human U133A 2.0 chips according to manufacturer's protocol. Biological duplicates were prepared for each condition. GC-RMA method was used to extract the gene expression values. Expression data were then normalized using quantile normalization. To select for genes whose expressions were downregulated by p65 knockdown, a >1.7-fold decrease cut-off was chosen when comparing expression data between ND KD and STK38/38L KD samples.

Gene symbol	Gene name
STK38L	serine/threonine kinase 38 like
STK38	serine/threonine kinase 38
TACSTD2	tumor-associated calcium signal transducer 2
(SAA1, SAA2)	serum amyloid A1, serum amyloid A2
INSIG1	insulin induced gene 1
FGFBP1	fibroblast growth factor binding protein 1
INSIG1	insulin induced gene 1
TRIM2	tripartite motif-containing 2
STK38	Serine/threonine kinase 38
ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6
HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
DYRK1A	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A
GIT2	G protein-coupled receptor kinase interactor 2
TRIM2	tripartite motif-containing 2
SCD	stearoyl-CoA desaturase
FZD7	frizzled homolog 7
IL18	interleukin 18
LTB	lymphotoxin beta
TNF	tumor necrosis factor alpha
GNS	glucosamine (N-acetyl)-6-sulfatase

Table-S-2.5 Genes upregulated by p65 knockdown. NS KD and p65 KD HCT116 cells were treated with 100 ng/mL TNF-α for 6 hours before mRNA samples were collected. cDNA was synthesized and labeled before hybridizing on the Affymatrix Human U133A 2.0 chips according to manufacturer's protocol. Biological duplicates were prepared for each condition. GC-RMA method was used to extract the gene expression values. Expression data were then normalized using quantile normalization. To select for genes whose expressions were downregulated by p65 knockdown, a >1.8-fold increase cut-off was chosen when comparing expression data between ND KD and p65 KD samples.

Gene symbol	Gene name
SETD2	SET domain containing 2
TSPAN5	tetraspanin 5
JUN	jun oncogene
RNASET2	ribonuclease T2
NOL5A	nucleolar protein 5A (56kDa with KKE/D repeat)
XPO4	exportin 4
SART3	squamous cell carcinoma antigen recognized by T cells 3
SLC43A3	solute carrier family 43 member 3
MAPK1	mitogen-activated protein kinase 1

Table-S-2.6 Genes upregulated by STK38/38L knockdown. NS KD and STK38/38L KD HCT116 cells were treated with 100 ng/mL TNF-α for 6 hours before mRNA samples were collected. cDNA was synthesized and labeled before hybridizing on the Affymatrix Human U133A 2.0 chips according to manufacturer's protocol. Biological duplicates were prepared for each condition. GC-RMA method was used to extract the gene expression values. Expression data were then normalized using quantile normalization. To select for genes whose expressions were downregulated by p65 knockdown, a >1.7-fold increase cut-off was chosen when comparing expression data between ND KD and STK38/38L KD samples.

Gene symbol	Gene name
MYCBP2	MYC binding protein 2
ZNF232	zinc finger protein 232
VCAN	versican
ADFP	adipose differentiation-related protein
BRD4	bromodomain containing 4
RBP1	retinol binding protein 1
FBXO42	F-box protein 42
LASS6	LAG1 homolog ceramide synthase 6
INTS7	integrator complex subunit 7
MSRB2	methionine sulfoxide reductase B2

qPCR primers

Target gene	Forward (5'- 3')	Reverse (5'- 3')
LTB	GACGAAGGAACAGGCGTTTCT	GTAGCCGACGAGACAGTAGAG
TNF	CCCAGGGACCTCTCTCTAATCA	GCTACAGGCTTGTCACTCGG
IκBα	CCGCACCTCCACTCCATCC	ACATCAGCACCCAAGGACACC
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

ChIP primers

Target promoter	Forward (5'- 3')	Reverse (5'- 3')
LTB	AGAGCCTGTCTCCCTAACCTC-AACTTC	CCTGTAGACCTGCACACCTGGC
TNF	TCTGGGAATTCCAATCCTTGC-TGGGAAA	TTAAGTGGCTGGTTCAGGTTG-CAGAGTT
IκBα	AAGGCTCACTTGCAGAGGGACAGG-ATTA	GGAATTTCCAAGCCAGTCAGA-CCAGAAA

Table-S-2.7 Primers for realtime PCR and ChIP (listed in the 5' to 3' direction).

Chapter 3: Investigating the role of pro-inflammatory cytokines in TRAIL-induced apoptosis

This chapter is being prepared for submission: Hung N. Nguyen, Mingsheng Zhang, Peter K. Sorger

3.1 INTRODUCTION

Tumorigenesis is a multi-step process where normal cells have undergone to acquire malignancy. Two of the most important events leading up to cancer are genomic instability and inflammation (Hanahan and Weinberg, 2011). Genomic instability occurs through the generation of random mutations including chromosomal rearrangements and rare genetic changes that allow cancer cells to survive, proliferate, and disseminate (Jackson and Bartek, 2009; Negrini et al., 2010). While genomic instability has been demonstrated as one of enabling characteristics of cancer development for some time, inflammation, a process normally activated by our body to protect us from harmful stimuli like pathogens, dead cells and irritants, has only been recognized to be an important characteristics of tumorigenesis in the past decade or so (DeNardo et al., 2010; Grivennikov et al., 2010).

Virtually every tumor has been observed to be surrounded by immune cells (Pages et al., 2010). Such immune responses were originally thought of as an attempt by the immune system to eradicate tumors, which is true in some cases. However, tumor-associated inflammation has been shown to have an unanticipated effect of enhancing tumorigenesis and progression. Inflammation can cause the release of cytokines to the tumor microenvironment that can facilitate proliferation, survival, angiogenesis, invasion, metastasis, and activation of epithelial-mesenchymal transition (DeNardo et al., 2010; Grivennikov et al., 2010; Qian and Pollard, 2010).

Two of the most pleiotropic pro-inflammatory cytokines are TNF-α and IL-1α. TNF-α and IL-1α family proteins are abundant at tumor sites and they represent potent molecules that may greatly influence the direction of the malignant process (Apte et al., 2006; Balkwill, 2006). Although TNF-α and IL-1α bind to different types of receptors which then recruit different

adaptor molecules, both TNF-α and IL-1α can activate a common pathway that involves the transcription factor NF-κB (Locksley et al., 2001; Weber et al., 2010). This pathway has dual actions in tumor promotion: first by preventing death of cells with malignant potential, and second by stimulating production of pro-inflammatory cytokines (Varfolomeev and Ashkenazi, 2004). The survival branch of NF-κB activation involves upregulation of negative regulators of apoptosis such as c-FLIP, Bcl-2 while the death branch is mediated through caspase 8 and via accumulation of intracellular reactive oxygen, sustained Jun N-terminal kinase activation and mitochondrial pathways (Karin and Lin, 2002).

Given the role of pro-inflammatory cytokines in cancer in general and in apoptosis in particular, when it comes to cancer treatment, one important question is how the presence of these molecules affects the effectiveness of a therapy. One promising anti-cancer drug is TRAIL (TNF-Related Apoptosis Inducing Ligand). TRAIL has attracted considerable attention owing to its selective killing of tumor cells while normal cells were largely protected (LeBlanc and Ashkenazi, 2003). In animal models, treatment with TRAIL by itself or in combination with other drugs resulted in suppression of tumor xenografts without causing systemic toxicity (Kelley and Ashkenazi, 2004; Walczak et al., 1999). Clinical trials using TRAIL have been reported for patients with non-small-cell lung cancer, non-Hodgkin's lymphoma and colorectal carcinoma (Fischer and Schulze-Osthoff, 2005). Despite its tumor specific killing, some tumors can survive TRAIL treatment, which presents a major barrier for the development of efficient therapies (Kurbanov et al., 2007; Morizot et al., 2010; Wang and El-Deiry, 2003). Resistance to TRAIL has been suggested to be due to increased levels of antiapoptotic proteins such as c-FLIP, Bcl-2, Bcl-xL and inhibitors of apoptosis proteins (IAPs) (Zhang and Fang, 2005). As many of these proteins are known targets of NF-κB and this transcription factor is activated by pro-

inflammatory cytokines that are present at tumor's sites, this suggests that there is a link between inflammation and TRAIL resistance.

In this work, we examined the role of pro-inflammatory cytokines in TRAIL-induced cell death. Using a panel of four human liver cell lines, we first asked how pre-treatment of TNF-α and IL-1α affects the cell death response by TRAIL. To determine whether the NF-κB pathway is responsible for the observed apoptotic outcomes, we used a dominant negative inhibitor of NF-κB. We then employed various approaches to find genes and proteins whose expressions are regulated by NF-κB and play a contributing role in the observed phenotypes.

3.2 RESULTS

3.2.1 Pro-inflammatory cytokine stimulations have different apoptotic effects on cell lines

Using a panel of four human liver cell lines which include two immortalized (HHL5 and HHL7) (Clayton et al., 2005) and two hepatocellular carcinoma (Hep3B and Focus), we examined the effect of pro-inflammatory cytokines including TNF-α and IL-1α on the apoptotic behaviors of these cell lines. To effectively assay for the apoptotic potential of these cytokines, we used superkiller TRAIL to induce cell death after they had been pre-treated with pro-inflammatory cytokines. In this experimental setting, cells were plated and starved overnight before being treated with 100 ng/mL TNF-α or 10 ng/mL IL-1α for 2 hours (referred to as 2HR condition), 4 hours (referred to as 4HR condition) and overnight (referred to as O-N condition), after which 100 ng/ml TRAIL was added for another 24 hours with the pre-treated cytokines still in the media. Media was then removed and cells were washed, fixed and stained with methylene blue in 50% ethanol. The amount of cells remained was quantified by absorbance spectrometry. With this approach, we were able to observe different apoptotic outcomes that were time,

stimulus and cell line-dependent (Figure 3.1). Specifically, for HHL5 cells, both TNF-α and IL-1α treatments conferred survival advantage against TRAIL killing. However, for HHL7 cells, only TNF-α and short-term IL-1α stimulations (2HR and 4HR) gave these cells pro-survival advantage while long-term IL-1α treatments (O-N) had no effects. For Focus cells, only pro-survival behavior of TNF-α was observed while IL-1α appeared to have no effects regardless of the duration of treatment. For Hep3B cells, both long-term treatments with TNF-α and IL-1α made these cells more prone to be killed by TRAIL. A summary of the diverse apoptotic behaviors of cells pre-treated with pro-inflammatory cytokines followed by TRAIL is presented in Figure 3.2. When cells were treated with only TNF-α or IL-1α without TRAIL, we observed very little apoptotic effects induced by these two cytokines (Figure S-3.1) except for HHL7 cells where only a prolonged treatment with TNF-α for about 48 hours (O-N) resulted in ~25% cell death. Apparently, treating cells with pro-inflammatory cytokines alone did not give us much information about the apoptotic behaviors of these cells.

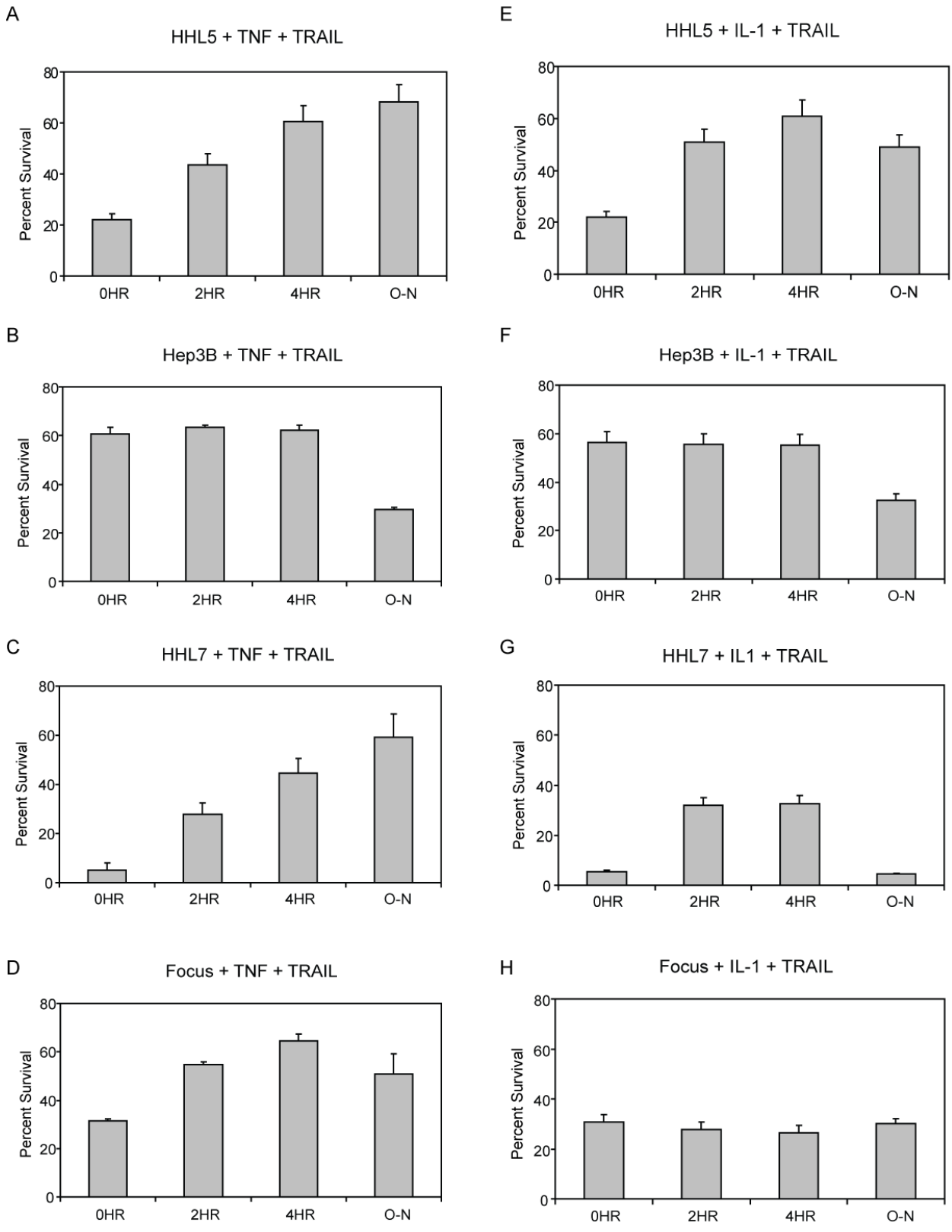

A
HHL5 + TNF + TRAIL
Percent Survival
0
20
40
60
80
0HR
2HR
4HR
O-N
E
HHL5 + IL-1 + TRAIL
Percent Survival
0
20
40
60
80
0HR
2HR
4HR
O-N
B
Hep3B + TNF + TRAIL
Percent Survival
0
20
40
60
80
0HR
2HR
4HR
O-N
F
Hep3B + IL-1 + TRAIL
Percent Survival
0
20
40
60
80
0HR
2HR
4HR
O-N
C
HHL7 + TNF + TRAIL
Percent Survival
0
20
40
60
80
0HR
2HR
4HR
O-N
G
HHL7 + IL1 + TRAIL
Percent Survival
0
20
40
60
80
0HR
2HR
4HR
O-N
D
Focus + TNF + TRAIL
Percent Survival
0
20
40
60
80
0HR
2HR
4HR
O-N
H
Focus + IL-1 + TRAIL
Percent Survival
0
20
40
60
80
0HR
2HR
4HR
O-N

Figure 3.1 Apoptotic responses of human liver cell lines to pro-inflammatory cytokine pre-treatment followed by TRAIL. (A, B, C, D) Responses of HHL5, Hep3B, HHL7 and Focus to TNF-α pre-treatments. Cells were starved overnight in serum free media. On day 1, cells were treated with 100 ng/mL TNF-α (referred to as O-N condition). On day 2, untreated cells were stimulated with 100 ng/ml TNF-α for 2 hours (referred to as 2HR condition) or 4 hours (referred to as 4HR condition) after which 100 ng/ml superkiller TRAIL was added to all TNF-α pre-treated cells (O-N, 2HR, 4HR) and no media was changed. On day 3, dead cells were washed off and remaining cells were fixed and stained with methylene blue in 50% ethanol. Surviving cells were quantified by absorbance spectrometry and normalized to untreated cells. (E, F, G, H) Responses to HHL5, Hep3B, HHL7 and Focus to IL-1α pre-treatments. Experimental procedures were as in (A, B, C, D), except cells were pre-treated with 10 ng/ml IL-1α. Error bars represent the standard deviation of the mean of biological duplicates.

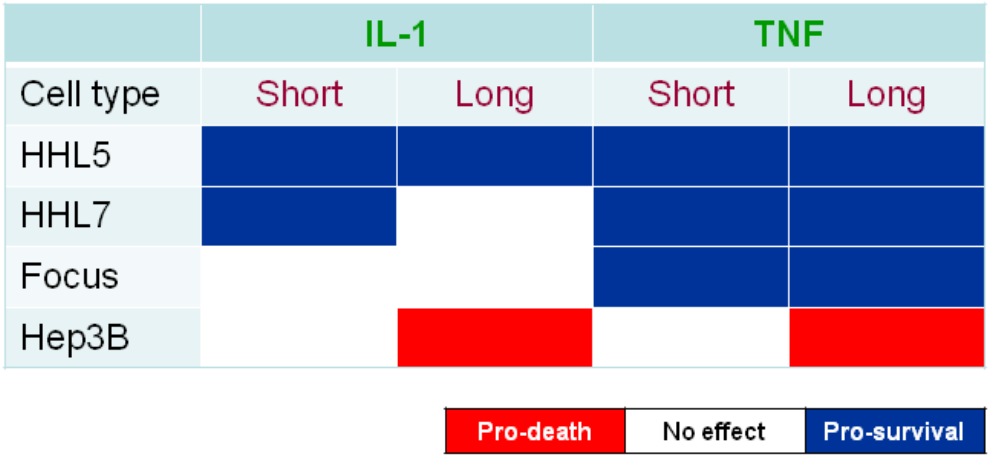


Figure 3.2 Summary of apoptotic responses of human liver cell lines to pro-inflammatory cytokine pre-treatment followed by TRAIL. Short represents the 2 and 4 hours cytokine stimulations while long represents the overnight stimulations.

3.2.2 NF-κB pathway is responsible for these diverse apoptotic outcomes

Since NF-κB pathway is known to be activated by pro-inflammatory cytokines, to examine whether this pathway is responsible for the diverse apoptotic outcomes described above,

we used a dominant negative inhibitor of NF-κB (referred to as IκB-DN). This inhibitor has two serine to alanine mutations (S32 and S36) (Boehm et al., 2007), which prevent it from being phosphorylated by an upstream kinase IKK and thus blocking the nuclear translocation of NF-κB and its activation. The inhibitor was introduced into cells by retroviral infection and cells with the inhibitor stably expressed were selected with puromycin.

Using cell lines carrying the stably expressed IκB-DN protein and employing the same experimental setting where cells were pre-treated with TNF-α or IL-1α for different durations and then with TRAIL, we asked whether blocking the activation of NF-κB pathway altered their apoptotic responses. For TNF-α stimulations, while treatment of this cytokine in the original HHL5 cells did not cause them to die (Figure S-3.1), the same treatment in the HHL5+IκB-DN killed as many cells as with the combined TNF-α and TRAIL treatments, suggesting that activation of the NF-κB pathway protects HHL5 cells from both TNF-α and TRAIL killing (Figure 3.3A, B). For IL-1α stimulations, both HHL5 and HHL5+IκB-DN cells showed no significant apoptotic effects. However, when treated with IL-1α and then TRAIL, only the original HHL5 cells exhibited resistance to TRAIL killing but not the HHL5+IκB-DN ones, further confirming that both TNF-α and IL-1α pre-treatments of HHL5 cells activated the NF-κB pathway which confers the observed pro-survival behaviors (Figure 3.3C, D). For TNF-α and IL-1α stimulations, neither of these pro-inflammatory cytokines caused Hep3B or Hep3B+IκB-DN cells to die (Figure 3.4A, C). However, when treated with TNF-α or IL-1α overnight followed by TRAIL, only Hep3B cells exhibited the pro-death behavior, which can be blocked in Hep3B+IκB-DN cells, suggesting that the NF-κB pathway plays a pro-death role in this cell line (Figure 3.4B, D). For the remaining two cell lines HHL7 and Focus, overexpressing an IκB-DN protein was enough to eliminate any effects of pro-inflammatory cytokines pre-treatments

(Figure S-3.2, S-3.3). Taken together, it is evident that activation of the NF-κB pathway is responsible for the diverse apoptotic behaviors observed in these four liver cell lines.

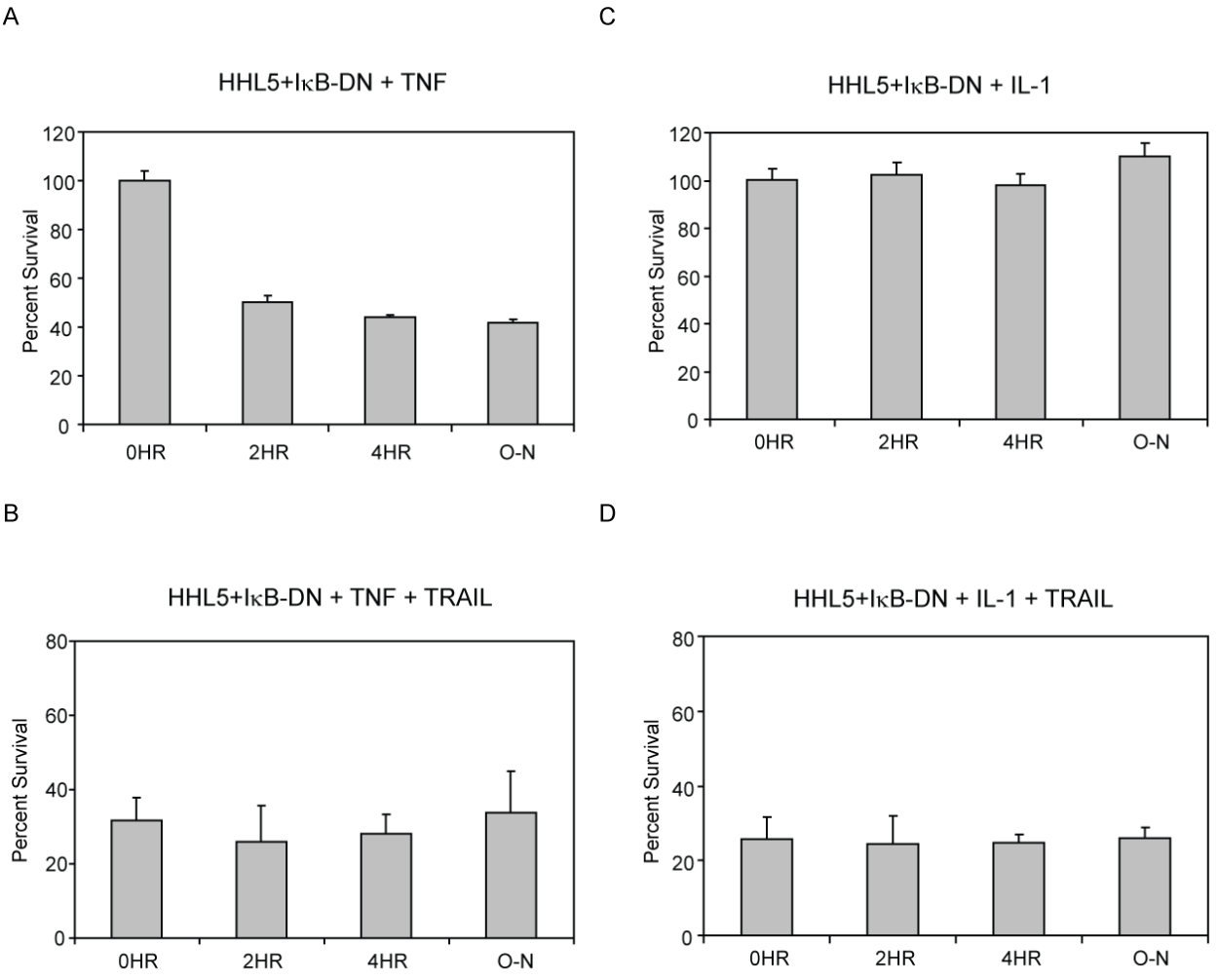


Figure 3.3 Apoptotic responses of HHL5+IκB-DN cells. (A, C) Responses of HHL5+IκB-DN cells to TNF-α and IL-1α alone. HHL5+IκB-DN cells were generated as described in the Materials and Methods section. Cells were starved overnight in serum free media. On day 1, cells were treated with 100 ng/mL TNF-α or 10 ng/ml IL-1α (referred to as O-N condition). On day 2, untreated cells were stimulated with 100 ng/ml TNF-α or 10 ng/ml IL-1α for 2 hours (referred to as 2HR condition) or 4 hours (referred to as 4HR condition). On day 3, dead cells were washed off and remaining cells were fixed and stained with methylene blue in 50% ethanol. Surviving cells were quantified by absorbance spectrometry and normalized to untreated cells. (B, D) Responses of HHL5+IκB-DN cells to TNF-α and IL-1α pre-treatments followed by TRAIL. Experimental procedures were as in (A, C), except on day 2, 100 ng/ml TRAIL was added. Error bars represent the standard deviation of the mean of biological duplicates.

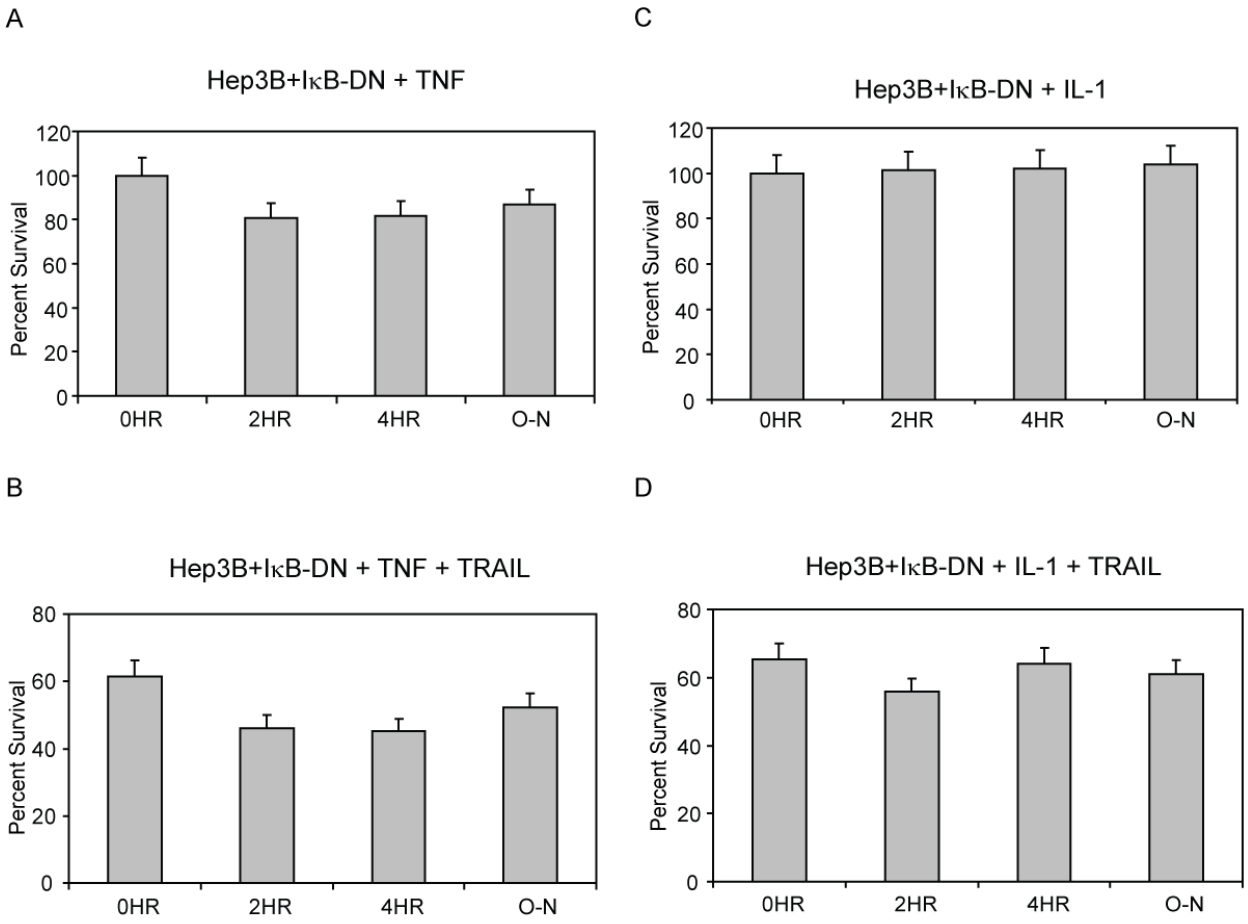


Figure 3.4 Apoptotic responses of Hep3B+IκB-DN cells. (A, C) Responses of Hep3B+IκB-DN cells to TNF-α and IL-1α alone. Hep3B+IκB-DN cells were generated as described in the Materials and Methods section. Experimental procedures were similar to those in (Figure 3.3A, C). (B, D) Responses of Hep3B+IκB-DN cells to TNF-α and IL-1α pre-treatments followed by TRAIL. Experimental procedures were as in (Figure 3.3B, D). Error bars represent the standard deviation of the mean of biological duplicates.

3.2.3 Changes in the level of various pro- and anti-apoptotic proteins in response to pro-inflammatory cytokines can help explain the phenotypic outcomes

As activation of the NF-κB pathway is responsible for diverse apoptotic responses, we sought to examine genes and proteins that have been implicated in the apoptotic pathway and whose expressions are under the control of the NF-κB transcription factor. To accomplish this,

we first collected NF-κB's promoter binding data by carrying out a chromatin immunoprecipitation followed by high throughput sequencing (ChIP-Seq) experiment. To perform a ChIP-Seq assay, cells were starved overnight and then treated with 100 ng/mL TNF-α for 6 hours before being fixed with 1% formaldehyde for 10 minutes. Chromatin was extracted and sonicated to obtain DNA fragments in the 200-500 bp range. NF-κB's bound promoters were immunoprecipitated with an antibody against its p65 subunit. The promoters' regions were sequenced by an Illumina high throughput sequencing machine, which can sequence up to 30 million reads per sample. We obtained the ChIP-Seq data from two hepatocellular carcinoma HepG2 and Focus as these data were already available to us. Because our primary goal for this step was to obtain as many promoters of genes that have a role in apoptosis, using data on HepG2 and Focus cells should suffice. Our sequencing results for HepG2 and Focus contained more than 13 million unique reads for each sample. From the promoters' sequence data, we used the QuEST program (Valouev et al., 2008) to identify the chromosomal regions bound by NF-κB's p65 in response to cytokine's stimulation. We then used a second program GREAT (McLean et al., 2010) to find genes whose regions around transcription start sites (2000 bp upstream and 1000 bp downstream) include the promoter's binding regions identified by QuEST (supplemental Table-S-3.1). For our purpose, we only focused on genes that function in apoptosis (Table 3.1A).

A.

Gene name
BAD
BID
BIRC2
BIRC3
CASP10
CASP4
CFLAR
CRADD
CYCS
MDM2
NFKBIB
NLRC4
RB1
RELA
STAT5A
TNFRSF10B
TP53
USP7

B.

Gene name
BAD
BID
BIRC2
BIRC3
CFLAR (FLIP)
NFKBIB
RELA
TNFRSF10B (DR5)

Table 3.1 Candidate pro- and anti-apoptotic genes. (A) Genes obtained from ChIP-Seq analsyis. ChIP-Seq experiment was carried out as described in the Materials and Methods section. Sequencing data from ChIP-Seq were used as input for the QuEST program (Valouev et al., 2008), which generated peaks (transcription factor's binding regions). The peaks data were then used as input for the GREAT program (McLean et al., 2010), which associated binding regions with genes and categorized them into functional groups. For our purpose, we focused on genes whose regions around transcription start sites (2000 bp upstream and 1000 bp downstream) contain the promoter's binding sites identified by QuEST and also function in the apoptosis pathway. (B) Genes with known apoptotic functions. From the list of genes in (A), we then used the NF-κB database (see http://www.bu.edu/nf-kb/) which contains known NF-κB induced target genes with established functional studies to select only genes that are also present in the database.

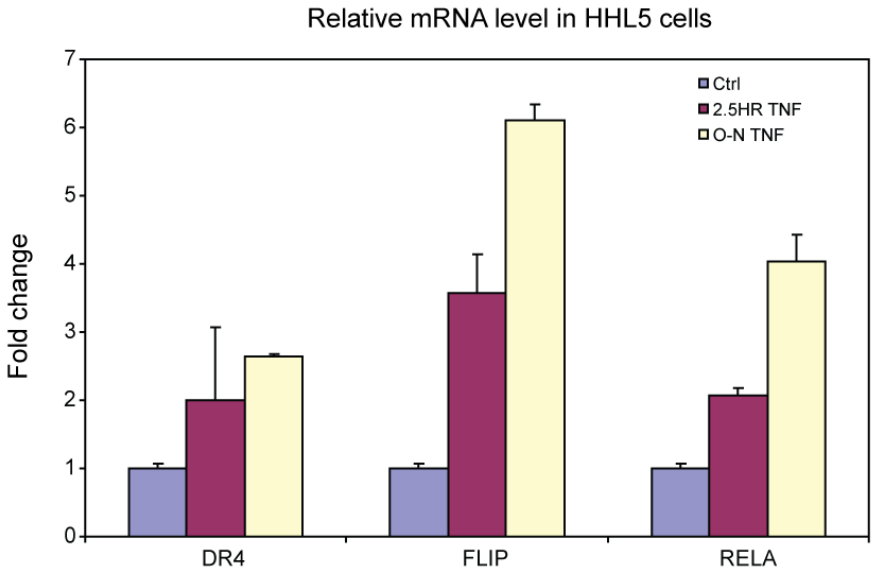
To reduce the number of genes in Table 3.1A, we then chose only genes that were also present in the NF-κB database (see http://www.bu.edu/nf-kb/) which contains known NF-κB target genes with established functional studies (Table 3.1B). From this narrowed down group of genes, we examined whether their expressions changed in response to pro-inflammatory cytokine stimulations. For this experiment, cells were starved overnight and then treated with 100 ng/ml

TNF-α for 2.5 hours (representing the early response) and overnight (representing the late response), after which RNA samples were collected. Real-time PCR was performed and changes in genes' expression were calculated as the fold-change in the qPCR signals between treated and untreated samples. Genes whose expressions showed at least two-fold changes in any stimulation conditions were presented (Figure 3.5). For HHL5 cells, in response to TNF-α treatments, upregulation of DR4, FLIP and RELA/p65 mRNA was observed. For Hep3B cells, TNF-α stimulations were seen to increase expression of BID, BAD, DR5 and FLIP. We also observed increases in expression of BIRC2 and BIRC3 genes in HHL5 and Hep3B cells (data not shown). However, since these genes appeared to function mainly as the pro-survival factors in the TNF-α induced cell death as their highest expression in Hep3B cells treated with TNF-α overnight could not prevent these cells from being killed by TRAIL, we decided not to include them in our study.

To further confirm the gene expression data, we measured the protein levels of these genes by Western blotting. To collect protein samples, cells were starved overnight and then treated with either 100 ng/mL TNF-α or 10 ng/mL IL-1α for 6 hours (representing the early response) and overnight (representing the late response). A BCA assay was carried out to quantify protein concentration and equal amounts of total protein were loaded for each sample. For HHL5 cells, we observed a very quick increase in the level of FLIP protein at the early time point and FLIP level stayed elevated at the late time point in response to both TNF-α and IL-1α stimulations (Figure 3.6A). A similar pattern for the NF-κB's p65 protein was observed. On the other hand, the level of the death receptor DR4 showed a significant reduction with the overnight treatment of TNF-α and IL-1α. The Western blot data confirmed the qPCR data, except for DR4, which will be discussed later. Also, changes in proteins' levels of FLIP, p65 and DR4 were not

observed in the HHL5+IκB-DN cells, indicating that the NF-κB pathway is responsible for these changes.

A



B

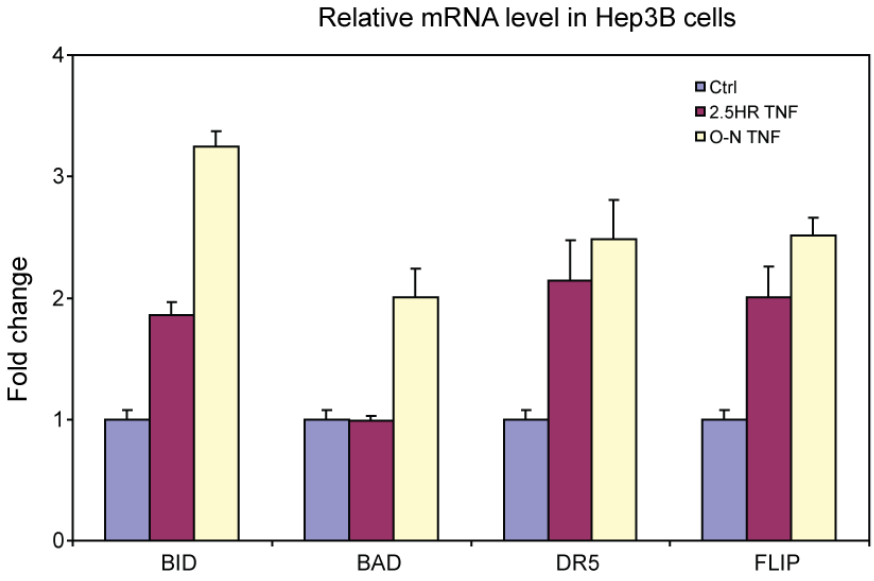


Figure 3.5 Plots of mRNA expression of pro- and anti-apoptotic genes in TNF-α treated cells. (A, B) Relative mRNA level in HHL5 and Hep3B cells. HHL5 cells were starved overnight. On day 1, cells were treated with 100 ng/ml TNF-α. On day 2, untreated cells were stimulated with 100 ng/ml TNF-α for 2.5 hours, after which mRNA samples were extracted for

realtime PCR. Fold change was calculated based on the difference in the number of cycles between the TNF-α -treated and untreated cells. Error bars represent the standard deviation of the mean of biological duplicates.

For Hep3B cells, we observed an increase in the level of several pro-death proteins including BID and BAD in response to TNF-α and IL-1α treatments while in the Hep3B+IκB -DN cells, such increase was effectively blocked (Figure 3.6B). This suggests that the NF-κB pathway plays a role in upregulating pro-apoptotic proteins in Hep3B cells which contributes to the observed pro-death behavior. For HHL7 cells, we observed similar changes in the level of FLIP, p65, DR4 and DR5 proteins when cells were treated with TNF-α as in HHL5 cells. However, when stimulated with IL-1α, only upregulation of FLIP and p65 was evident for the early response but no significant downregulation of DR4 and DR5 was observed at the late time point, which correlates well with the phenotypic outcomes described for this cell line when treated with IL-1α as this cytokine only induced a short-term pro-survival effects. For Focus cells, when treated with TNF-α, we observed similar changes in FLIP, p65, DR4 and DR5 protein but to a smaller extent. However, IL-1α did not appear to produce any responses in this cell line at the phenotypic level or signaling or ChIP-Seq (data not shown), which suggests that Focus cells have no or non-functional IL-1α receptor.

To examine how the protein levels compare across all four cell lines, we loaded the same amounts of total protein for each sample and run them on the same Western blots (Figure 3.7). For DR4 protein, Focus, HHL5 and HHL7 expressed it at similar levels but Hep3B cells appeareed to lack this receptor. When cells were treated with TNF-α, HHL5 and HHL7, however, showed significantly more downregulation of this receptor compared to Focus. For

BID and BAD protein, Hep3B cells showed much higher expression of these pro-death proteins compared to the other three. For FLIP, this protein was upregulated strongly in HHL5 and HHL7 in response to TNF-α and IL-1α.

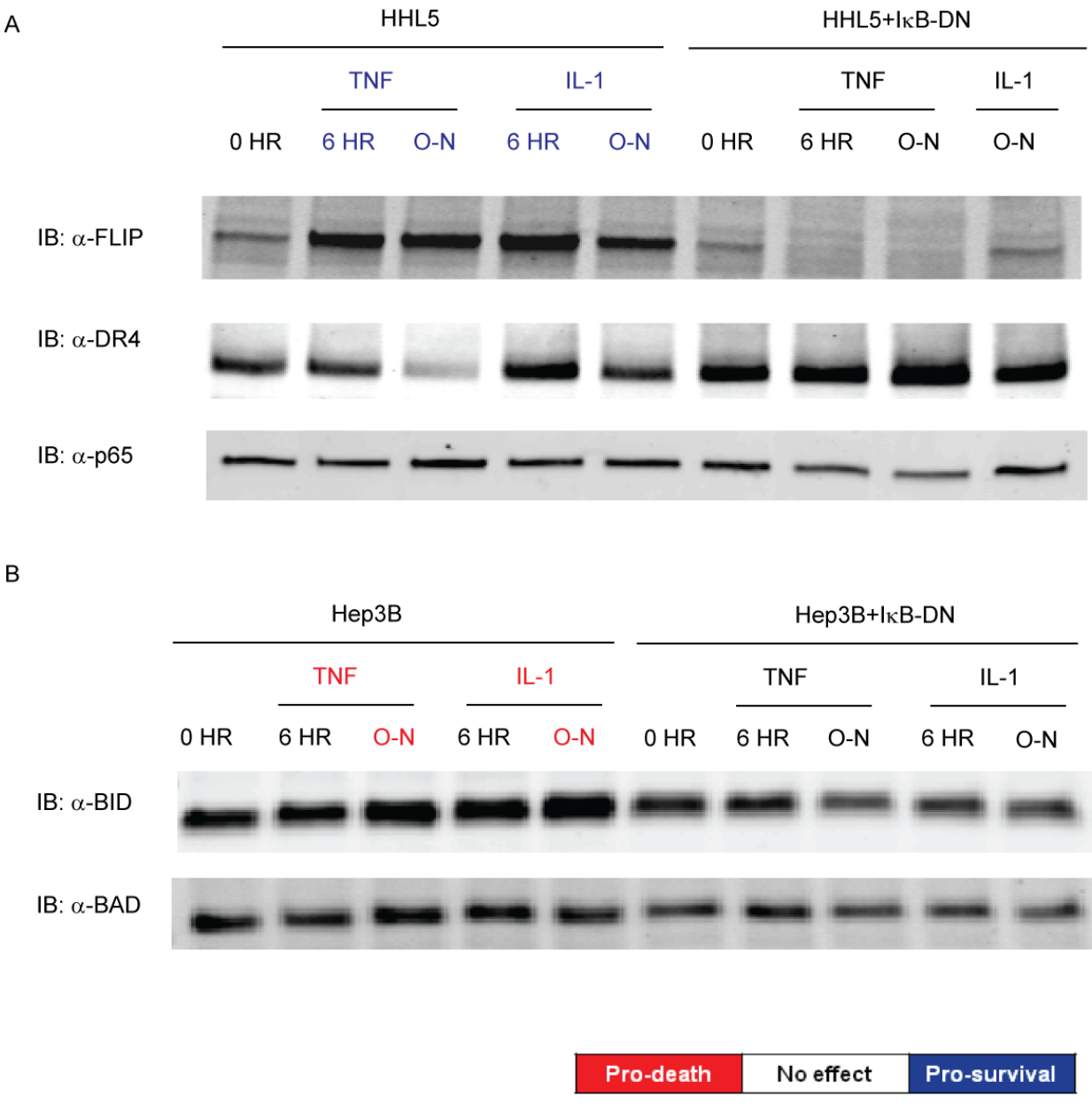


Figure 3.6 Western blots of pro- and anti-apoptotic proteins in TNF-α and IL-1α treated cells. (A) Western blots of FLIP, DR4 and p65 in HHL5 and HHL5+IκB-DN cells. Cells were starved overnight. On day 1, cells were treated with 100 ng/ml TNF-α or 10 ng/ml IL-1α (referred to as O-N condition). On day 2, untreated cells were stimulated with 100 ng/ml TNF-α or 10 ng/ml IL-1α for 6 hours, after which whole cell lysates were collected for Western blots. A BCA assay was performed to determine protein concentration and equal amounts of total protein were loaded for each sample. (B) Western blots of BID and BAD in Hep3B and Hep3B+IκB-DN cells. Experimental procedures were as in (A).

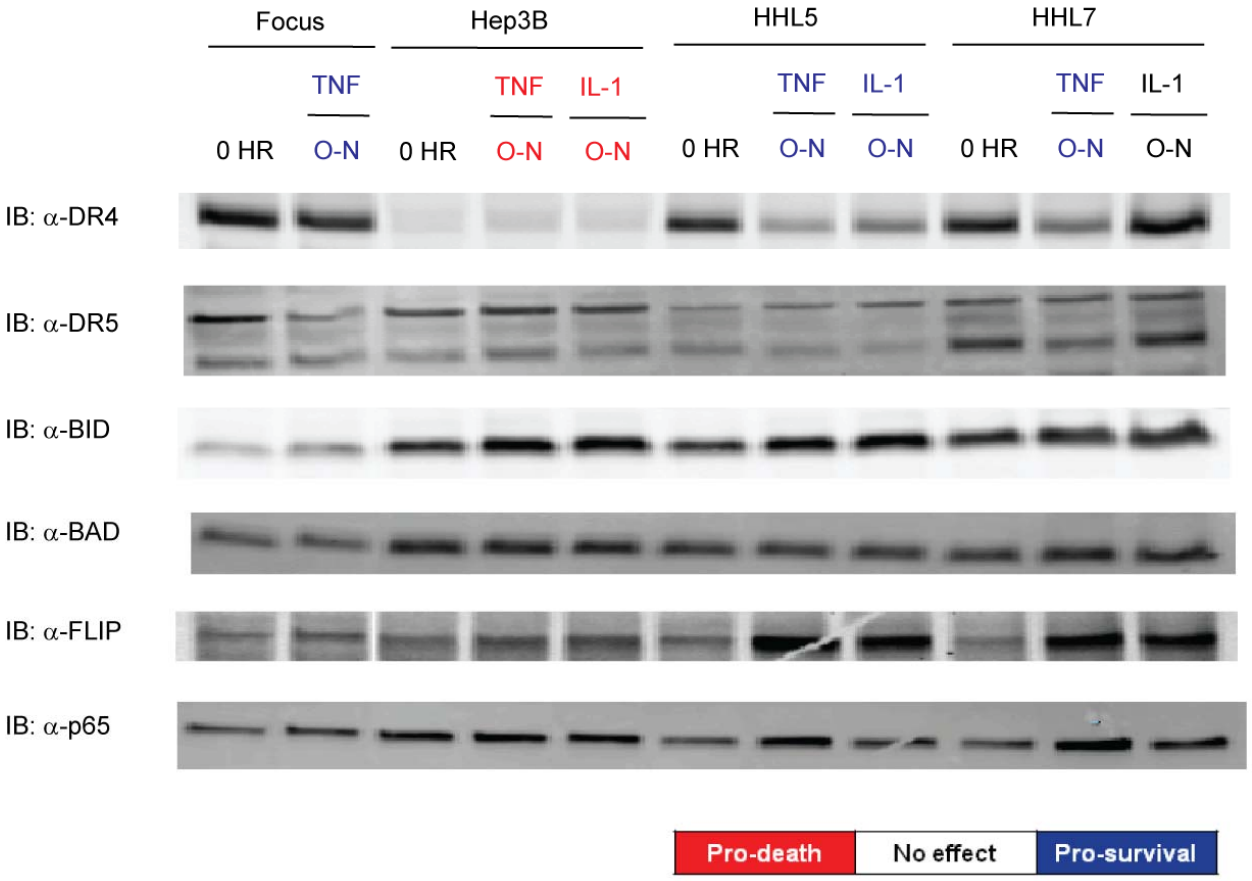


Figure 3.7 Western blots of pro- and anti-apoptotic proteins across four cell lines. Cells were starved overnight. On day 1, cells were treated with 100 ng/ml TNF-α or 10 ng/ml IL-1α (referred to as O-N condition). On day 2, whole cell lysates were collected for Western blots. A BCA assay was performed to determine protein concentration and equal amounts of total protein were loaded for each sample. Protein levels of DR4, DR5, BID, BAD, FLIP and p65 were measured. For Focus, since these cells did not response to IL-1α, only TNF-α treated samples were collected for this cell line.

3.3 DISCUSSION

In this study, we examined the pro-death and pro-survival potential of several human liver cell lines in response to pro-inflammatory cytokine treatments. We observed that pro-inflammatory cytokines had different effects on the apoptotic outcomes of these cell lines when cells were pre-treated with either TNF-α or IL-1α followed by TRAIL. While HHL5 cells showed pro-survival effects with both TNF-α and IL-1α stimulations, HHL7 exhibited only pro-survival behavior with TNF-α and short-term IL-1α treatments and Focus appeared to have only pro-survival response to TNF-α but not IL-1α. On the opposite end, Hep3B only displayed a pro-death phenotype with both TNF-α and IL-1α. We then demonstrated that the NF-κB pathway was responsible for the diverse apoptotic outcomes induced by pro-inflammatory cytokines TNF-α and IL-1α as introduction of a dominant negative inhibitor of NF-κB (IκB -DN) into these cells effectively blocked such outcomes.

To investigate how activation of NF-κB gave rise to diverse phenotypic outcomes, we took several approaches. We first used ChIP-Seq data collected for two hepatocellular carcinomas HepG2 and Focus cells to obtain promoters' regions bound by the NF-κB's p65 subunit in response to TNF-α. Next, we looked for candidate genes that lie close to these promoter regions and also have known functions in apoptosis. To further narrow the list of candidate genes, we only included genes which are also listed in the NF-κB database with existing functional studies. We then performed qPCR to measure changes in expression of these candidate genes in HHL5 and Hep3B cells treated with TNF-α. Genes that showed significant changes in their expression were selected for follow-up Western blotting. From the Western blot data, we observed that cells with pro-survival behaviors in response to TNF-α and IL-1α do so by upregulating p65 and FLIP and downregulating DR4 and DR5. The upregulation of p65 and

FLIP is more immediate, suggesting that this event is likely responsible for the early pro-survival phenotype whereas the downregulation of DR4 and DR5 occurs after a prolonged treatment of pro-inflammatory cytokines, implying that this event is more important in determining the late pro-survival behavior. For Hep3B cells that exhibit the pro-death response, upregulation of several apoptotic proteins including BID and BAD appears to play a contributing role.

Our results showed that TNF-α and IL-1α pre-treatments resulted in diverse apoptotic responses in four human liver cell lines, which suggests that there are underlying differences in their cellular networks. Since the NF-κB pathway was responsible for the various effects caused by TNF-α and IL-1α, this implies that activation of this pathway and the ensuing transcriptional programs are context (stimulus, time and cell type) dependent. Thus, using this type of context dependent approach will help us study different gene expression programs produced by the same transcription factor and also allow us to discover possibly new regulatory mechanisms. For example, in HHL5 and HHL7 cells, when treated with TNF-α overnight, we observed a sharp decrease in the level of DR4 and DR5 receptor and degradation products of these receptors were also evident on Western blots (Figure 3.8). On the mRNA level, when HHL5 cells were treated with TNF-α, expression of DR4 actually increased (Figure 3.5A) but we only observed an increase in this receptor at the protein level when the NF-κB was blocked (Figure 3.6A). The most likely explanation for this is when HHL5 and HHL7 cells are treated with TNF-α, expression of DR4, which appears to be independent of NF-κB, goes up but at the same time there is also an active degradation process that is dependent on the activation of NF-κB. In fact, it has recently been shown that DR4 and DR5 are subject to degradation by a ubiquitin-mediate process involving an E3-ubiquitin ligase c-Cbl (Song et al., 2010). This E3 ligase, however, is not an NF-κB's target gene, so in our case there may be a different E3 ligase involved. Ongoing

efforts are underway to identify additional DR4 and DR5's E3 ligases in HHL5 and HHL7 cells treated with TNF-α and IL-1α.

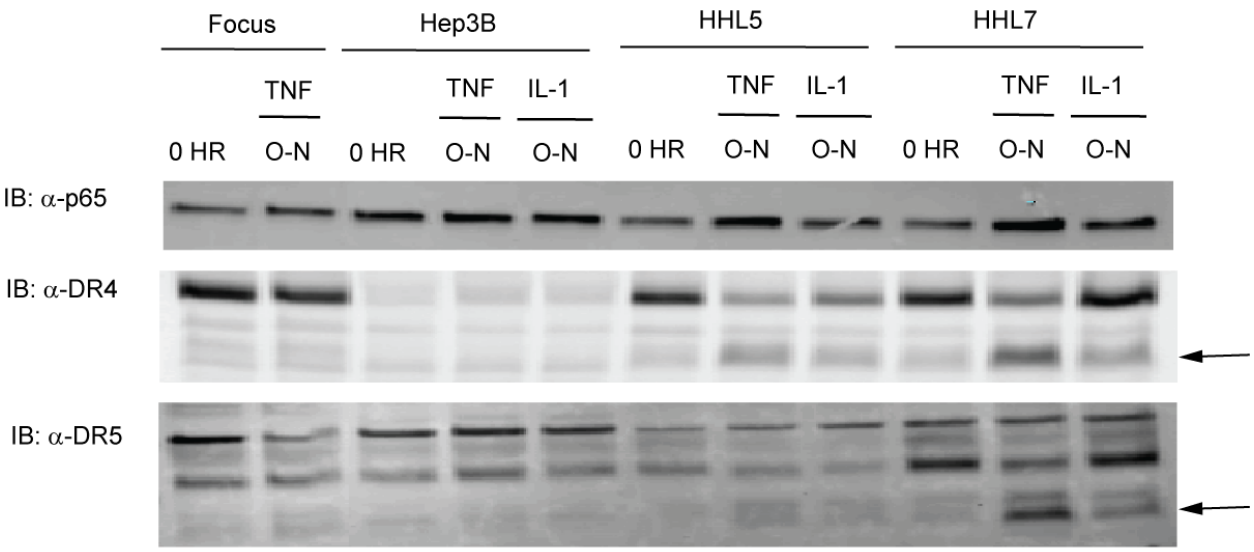


Figure 3.8 Degradation products of DR4 and DR5. Western blots were performed as described in Figure 3.7. However, bands smaller than DR4 and DR5 molecular weight were also included, indicated by arrows. These bands most likely represent the degradation products of DR4 and DR5 in HHL5 and HHL7 treated with TNF-α and IL-1α.

Also, by using a context dependent approach, we were able to observe the pro-death behavior of Hep3B cells treated with either TNF-α or IL-1α. Since the NF-κB pathway was again shown to be responsible for such behavior, it implies that activation of this pathway in Hep3B cells actually has a pro-death effect, which is a less understood phenomenon as NF-κB is thought to be mostly pro-survival. The pro-death effect of NF-κB was described as through the expression of death ligands such as FasL (Ho et al., 2011). However, treatment of Hep3B cells with both FasL and TRAIL together did not result in more cell death than treatment with TRAIL alone (data not shown). We believe that upregulation of several death proteins such as BID and BAD by NF-κB is directly responsible for the pro-death outcome of this cell line. In addition, we

also noticed that Hep3B cells show no or little expression of the DR4 receptor. Normally when cells with both DR4 and DR5 receptor are treated with TRAIL, these receptors form a heterotrimer. However, if one type of receptor is missing as in Hep3B, only a homotrimer of DR5 can be formed. It has been suggested that formation of different types of receptor's trimers upon TRAIL stimulation can have different apoptosis outcomes (Lemke et al., 2010). Therefore, it will be interesting to examine cells having only one type of death receptor and whether this can contribute to altering the death response of those cells as seen for Hep3B.

The NF-κB pathway is known to be involved in activation of many genes. In our study, we only showed several genes and proteins with the most likely contribution to the overall apoptotic behaviors of various cell types based on the correlative changes in their expressions and the resulting phenotypes. However, this does not rule out the involvement of other genes and proteins regulated by NF-κB. We are conducting a large scale expression profiling of these four cell lines in response to TNF-α and IL-1α using a Luminex bead-based mRNA measurement approach. This method can profile up to 1000 target gene expression per condition and can also process a large number of samples. We hope that with these types of data, we can uncover additional genes and proteins that not only play a role in the apoptosis pathway examined here but also other important cellular processes.

In vitro overexpression of p65 resulted in upregulation of FLIP and downregulation of DR4 (Chen et al., 2003). In our case, we also observed upregulation of FLIP and downregulation of DR4 together with upregulation of p65 in HHL5 and HHL7 in response to TNF-α and IL-1α in vivo. The increase in FLIP and the decrease in DR4 level were much more pronounced compared to that in Focus cells, where p65 was not upregulated significantly. This suggests that the dramatic changes in FLIP and DR4 protein levels in HHL5 and HHL7 cells are likely due to

the upregulation of p65. However, it is worth noting that at the protein level, p65 was only upregulated by about ~25% while at the mRNA level, p65's mRNA was increased anywhere from 2-4 fold. Apparently, activation of the NF-κB pathway in HHL5 and HHL7 also regulates the expression of p65, which has not been usually the case. It is therefore interesting to see what cause the differences in p65's regulation in HHL5 and HHL7 cells.

Inflammation in general and pro-inflammatory cytokines in particular play an important role in the function of our immune system. In addition, it has been observed that most tumors' microenvironments are present with immune cells. Inflammation surrounding tumors has been suggested to confer many necessary properties for the growth and development of those tumors such as proliferation, angiogenesis as well as metastasis. Other important role of inflammation and pro-inflammatory cytokines involves apoptosis. As demonstrated in our study, pro-inflammatory cytokines can potentiate very different apoptotic outcomes depending on the type of cytokines, time and cellular context. This has at least one important implication regarding cancer therapy using TRAIL. Due to its ability to kill tumors selectively, TRAIL has been used in clinical trials as a promising anti-cancer drug. However, our study highlights the importance of studying the microenvironment when using TRAIL as some pro-inflammatory conditions can result in TRAIL resistance.

Here, we have demonstrated that using a context dependent approach, we were able to reveal the complexity in the apoptotic behaviors of cells in response to pro-inflammatory cytokines. Starting from observations in apoptotic behaviors of cells pre-treated with pro-inflammatory cytokines followed by TRAIL induced cell death, we set out to investigate the changes in gene and protein expression using various approaches. This helps us gain a better insight into the diversity, flexibility and dynamics nature of cellular responses, which gives us

additional clues into various mechanisms cells employ, be it receptor degradation or death receptor-specific apoptotic responses. The more we understand about the cellular programs and networks and their working mechanisms, the better we are at predicting their behaviors and ultimately at coming up with strategies to intervene and treat diseases.

3.4 MATERIALS AND METHODS

Cell lines and Reagents

Human liver cell lines, HHL5, HHL7, Hep3B and Focus were cultured in Eagle's minimum essential medium (ATCC) plus 10% FBS, L-glutamine (Gibco), 1% penicillin/streptomycin and 20 mM HEPES. HEK 293T cells were cultured in DMEM with the same supplements as above. HHL5 and HHL7 cells were obtained from the Patel laboratory (University of Oxford, UK). HepG2 and Hep3B cells were purchased from ATCC, FOCUS cells were obtained from J. Wands (Brown University). The following antibodies were used: anti-p65 (sc-109, sc-372 from Santa-Cruz); anti-FLIP (clone NF6, a gift from I. Lavrik, Heidelberg, Germany); anti-DR4 and DR5 (ProSci); anti-BID (Atlas Antibody); anti-BAD (sc-8044, Santa-Cruz); anti-GAPDH (from Abcam). Other reagents were purchased from the following vendors: TNF-α and IL-1α from PeproTech; SuperKiller TRAIL (Alexis Biochemicals), SYBR reaction mix from Applied Biosystems.

Pro-inflammatory cytokines and TRAIL treatment

Cells were seeded in 24-well plates at density of ~25%. The following day, media was changed to serum-free media to starve cells overnight. The next day (day 1), 100 ng/mL TNF-α or 10 ng/mL IL-1α was added for the overnight (O-N) treatment. On day 2, 100 ng/mL TNF-α or 10 ng/mL IL-1α was added for 2 hours (2HR) or 4 hours (4HR) treatment followed by 100 ng/mL TRAIL. On day 3, media and dead cells were removed, remaining cells were washed once with PBS and fixed and stained with 5 mg/mL Methylene Blue (Sigma) in 50% ethanol for 30 minutes at room temperature. Cells were then washed 3 times with H2O and air-dried.

Cell viability assay

To quantify cells on plates, 10 mg/ml sarcosine (Sigma) in PBS was added to the air-dried cells for 30 minutes at room temperature. A duplicate of 100 µL solution from each well was transferred to two wells in a 96-well plate. Absorbance was read at 620 nm and 405 nm wavelength on a Victor^{3}V Plate reader (Perkin Elmer) (protocol adapted from (Oliver et al., 1989)). Fraction of cell surviving (percent survival) was calculated as the ratio of (Abs@620-Abs@405) for a treated sample divided by (Abs@620-Abs@405) for an untreated control.

Generation of cells expressing IκB-DN protein

293T cells were transfected with a pBabe-Puro-IκBalpha-mut (super repressor) (Addgene, Plasmid 15291) (Boehm et al., 2007) and pCL-ampho using Lipofectamine 2000 (Invitrogen). Media was changed 4 hours post transfection. Viruses were collected after 24 and 48 hours. HHL5, HHL7, Hep3B and Focus cells were infected with viruses for 24 hours. Cells stably expressing the IκB-DN proteins were selected using 1-4 µg puromycin (Invitrogen).

Chromatin Immunopreciptation – high throughput Sequencing (ChIP-Seq)

HepG2 and Focus cells were treated with TNF-α for 60 minutes followed by fixation with 10% formaldehyde (1% final concentration) for 10 minutes. Cells were lysed first in swelling buffer for 15 minutes (25mM HEPES pH 7.8, 1.5 mM $MgCl_2$, 10mM KCl, 0.1% NP-40, 1mM DTT, protease inhibitors (leupeptin, pepstatin, chymostatin) then in sonication buffer (50mM HEPES pH 7.8, 140mM NaCl, 1mM EDTA, 0.1% SDS, 1% TritonX-100 supplemented with protease inhibitors (leupeptin, pepstatin, chymostatin). Samples were sonicated for 3 minutes with 20-second pulse intervals and 1 minute off at each interval at 4^{o}C using a Fisher-Scientific 550 Sonic Dismembrator with output setting at 4. Sonicated DNA fragment sizes were between 200-500bp. Chromatin was immunoprecipitated using an anti-p65 (sc-372 Ab from Santa-Cruz) and Dynabead Protein A (Invitrogen) at 4^{o}C overnight. DNA was purified using

phenol/chloroform precipitation method. To prepare DNA for sequencing, adaptor oligos from the ChIP-Seq Sample Preparation Kit (llumina) were ligated to the ChIPed DNA per the manufacturer's protocol. Samples were then submitted for high through sequencing on a Illumina HiSeq 2000 at the BioMicro Center at the Massachusetts Institute of Technology.

Quantitative real-time PCR

mRNA samples were extracted from cells using the NucleoSpin RNA II kit (Macherey-Nagel). cDNA synthesis was done using RETROscript kit with oligo (dT) (Applied Biosystems). qPCR reactions were performed in duplicate by using SYBR Green PCR Master Mix (Applied Biosystems) in the Eppendorf Realplex Mastercycler S machine. Primers are listed in supplementary Table S-3.2. The relative transcription level was calculated by using the ΔΔCt method with GAPDH as a normalization control.

Western Blotting

Total cell lysates were collected by first washing cells once with cold PBS followed by adding lysate buffer (50mM HEPES pH 7.5, 10% glycerol, 100 mM NaCl, 0.1% SDS, 1% NP-40, supplemented with protease inhibitors leupeptin, pepstatin, chymostatin and phosphatase inhibitors sodium orthovanadate, sodium fluoride, and beta-glycerol phosphate). Cells were lysed for 30 minutes on ice followed by centrifugation at 15000g for 15 minutes at 4^0C. Protein concentration was measured by a microBCA kit (Pierce). Equal amounts of total protein were loaded and run on a 8% SDS-PAGE gel. Separated proteins were transferred onto a nitrocellulose membrane which was then blocked with Odyssey Blocking Buffer (Li-COR) and probed with primary antibodies overnight at 4^0C. Detection was performed using secondary antibodies conjugated with IRDye 680 or 800 (Rockland) using a Li-COR Odyssey Scanner.

3.5 REFERENCES

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3.6 SUPPLEMENTAL INFORMATION

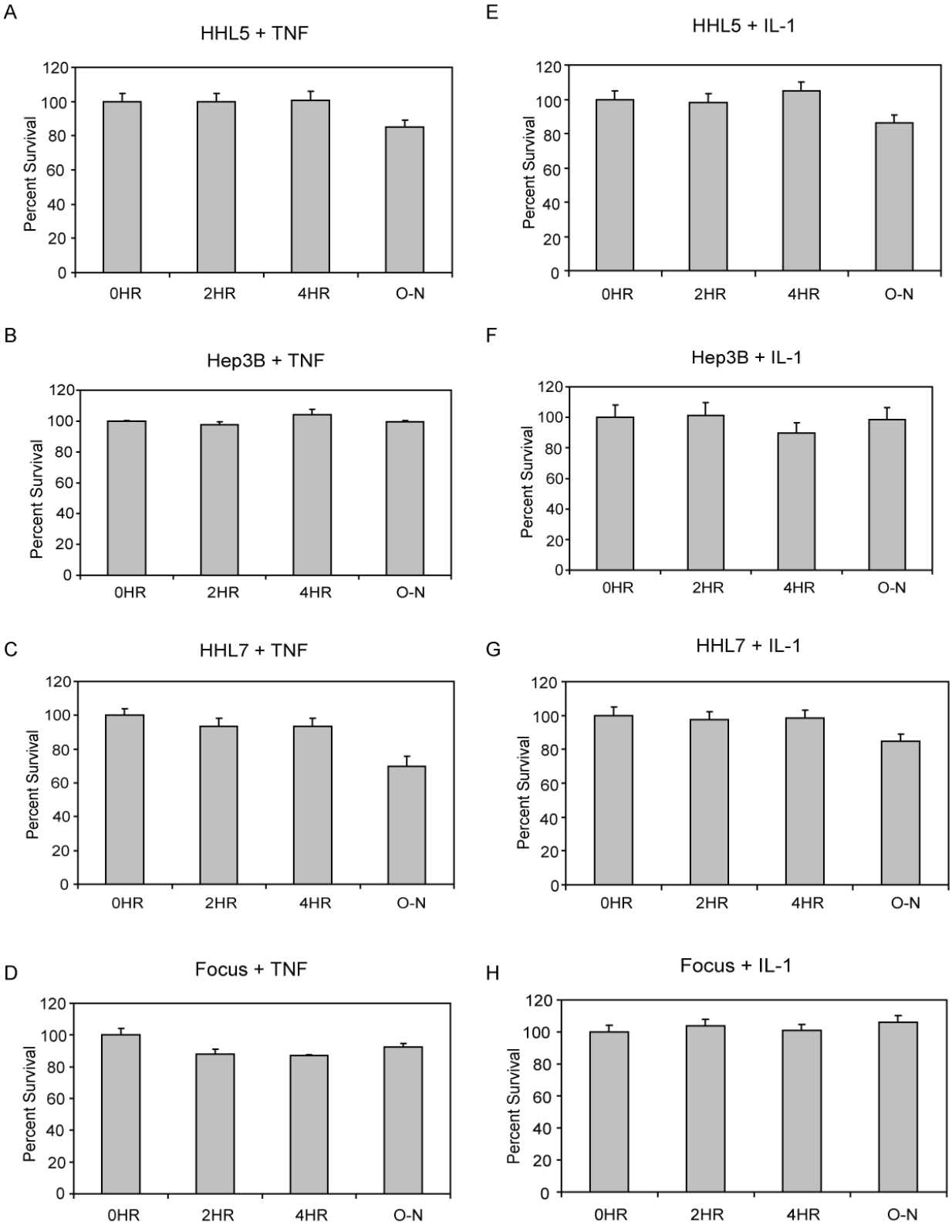

Figure S-3.1 Apoptotic responses of human liver cell lines to pro-inflammatory cytokines. (A, B, C, D) Responses of HHL5, Hep3B, HHL7 and Focus to TNF-α treatments. Cells were starved overnight in serum free media. On day 1, cells were treated with 100 ng/mL TNF-α (referred to as O-N condition). On day 2, untreated cells were stimulated with 100 ng/ml TNF-α for 2 hours (referred to as 2HR condition) or 4 hours (referred to as 4HR condition). On day 3, dead cells were washed off and remaining cells were fixed and stained with methylene blue in 50% ethanol. Surviving cells were quantified by absorbance spectrometry and normalized to untreated cells. (E, F, G, H) Responses to HHL5, Hep3B, HHL7 and Focus to IL-1α pre-treatments. Experimental procedures were as in (A, B, C, D), except cells were pre-treated with 10 ng/ml IL-1α. Error bars represent the standard deviation of the mean of biological duplicates.

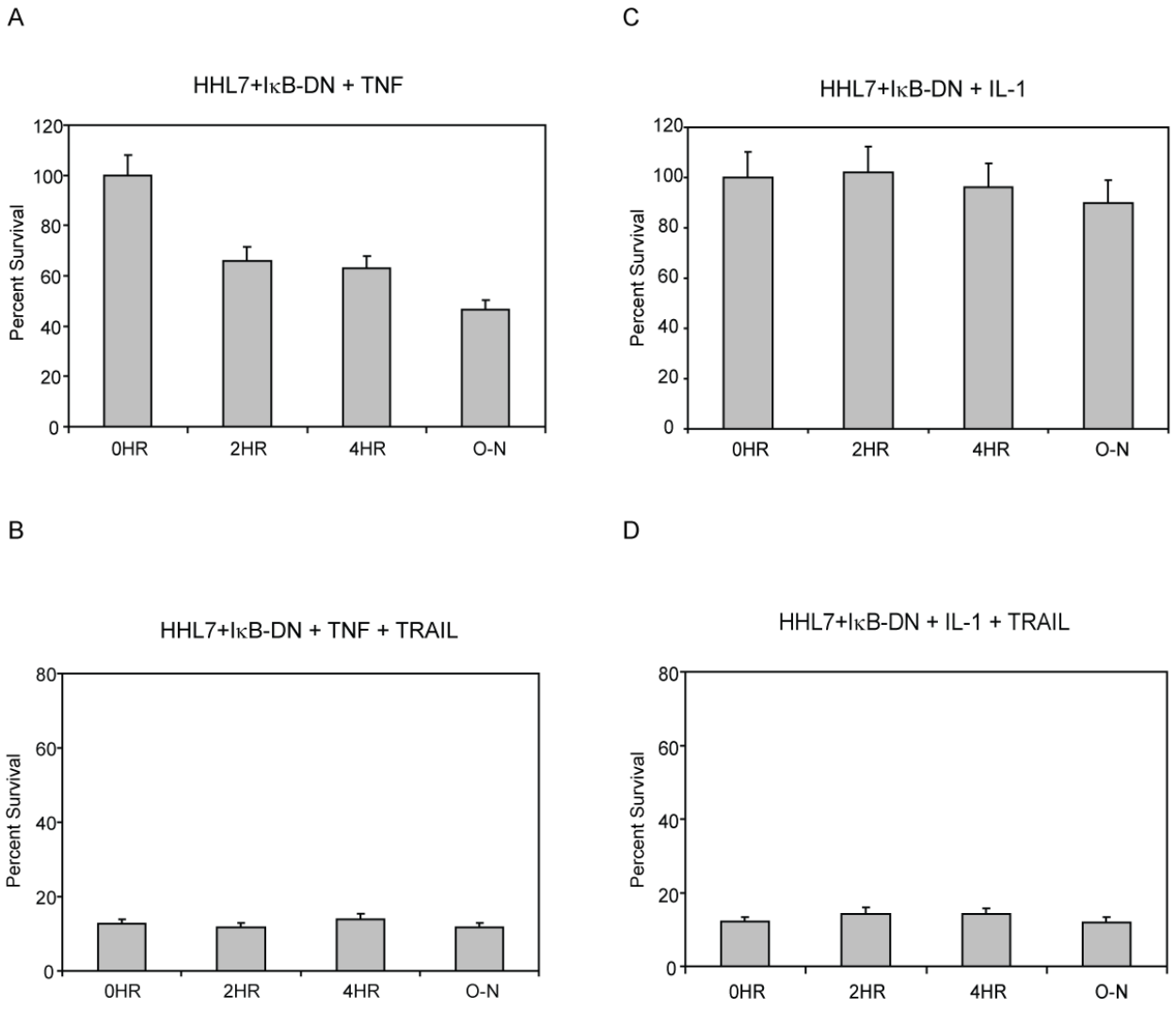

Figure S-3.2 Apoptotic responses of HHL7+IκB-DN cells. (A, C) Responses of HHL7+IκB-DN cells to TNF-α and IL-1α alone. HHL7+IκB-DN cells were generated as described in the Materials and Methods section. Cells were starved overnight in serum free media. On day 1, cells were treated with 100 ng/mL TNF-α or 10 ng/ml IL-1α (referred to as O-N condition). On day 2, untreated cells were stimulated with 100 ng/ml TNF-α or 10 ng/ml IL-1α for 2 hours (referred to as 2HR condition) or 4 hours (referred to as 4HR condition). On day 3, dead cells were washed off and remaining cells were fixed and stained with methylene blue in 50% ethanol. Surviving cells were quantified by absorbance spectrometry and normalized to untreated cells. (B, D) Responses of HHL7+IκB-DN cells to TNF-α and IL-1α pre-treatments followed by TRAIL. Experimental procedures were as in (A, C), except on day 2, 100 ng/ml TRAIL was added. Error bars represent the standard deviation of the mean of biological duplicates.

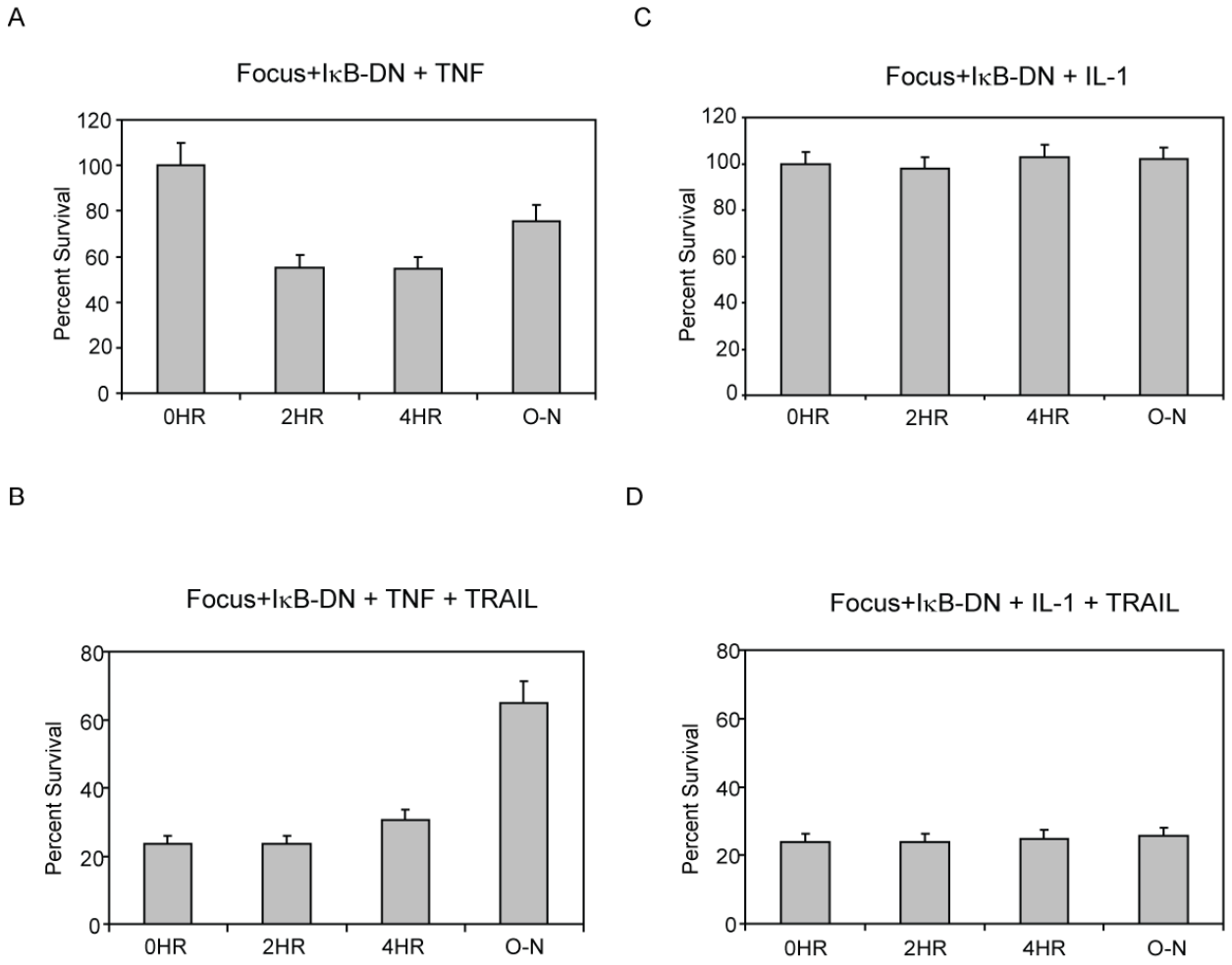

Figure S-3.3 Apoptotic responses of Focus+IκB-DN cells. (A, C) Responses of Focus+IκB-DN cells to TNF-α and IL-1α alone. Focus+IκB-DN cells were generated as described in the Materials and Methods section. Experimental procedures were similar to those in (Figure S-3.2A, C). (B, D) Responses of Focus+IκB-DN cells to TNF-α and IL-1α pre-treatments followed by TRAIL. Experimental procedures were as in (Figure S-3.2B, D). Error bars represent the standard deviation of the mean of biological duplicates.

Table S-3.1 Gene promoters bound by p65 in response to TNF-α stimulation. HepG2 and Focus cells were treated with TNF-α for 60 minutes followed by fixation with 10% formaldehyde (1% final concentration) for 10 minutes. Chromatin was immunoprecipitated using an anti-p65 (sc-372 Ab from Santa-Cruz) and Dynabead Protein A (Invitrogen) at 4^{o}C overnight. DNA was purified using phenol/chloroform precipitation method. To prepare DNA for sequencing, adaptor oligos from the ChIP-Seq Sample Preparation Kit (llumina) were ligated to the ChIPed DNA per the manufacturer's protocol. From the promoters' sequence data, the QuEST program (Valouev et al., 2008) was used to identify the chromosomal regions bound by NF- κB's p65 in response to cytokine's stimulation. A second program GREAT (McLean et al., 2010) was employed to find genes whose regions around transcription start sites (2000 bp upstream and 1000 bp downstream) include the promoter's binding regions identified by QuEST.

Gene name	Gene name	Gene name	Gene name
A2LD1	FBXO46	MREG	SESTD1
AADACL4	FBXW4	MRPL14	SF3B14
ABCA1	FCF1	MRPL19	SF3B2
ABCC11	FCHSD2	MRPL27	SFRS12IP1
ABCC4	FDPS	MRPL34	SFRS15
ABI1	FDXR	MRPL39	SFRS18
ABTB2	FERMT2	MRPS10	SFT2D2
ACAP2	FGF11	MRPS18B	SFT2D3
ACAT2	FGF2	MRPS24	SH2B3
ACP2	FGF6	MRPS33	SH3PXD2A
ACSS1	FGF7	MRPS9	SHH
ACSS3	FJX1	MSGN1	SIAH1
ACTR8	FKBP10	MSH5	SIRPB1
ADA	FKBP1B	MSH6	SIRT2
ADAM17	FLII	MT1B	SIRT4
ADAMTS9	FLJ16165	MT1F	SIX4
ADCK4	FLJ21075	MTF2	SKIL
ADCK5	FLJ21511	MTHFR	SKP2
ADFP	FLJ41766	MTMR14	SLC11A2
ADM2	FLOT2	MUC2	SLC15A1
ADPRH	FMNL1	MUC5AC	SLC16A6
AFMID	FMNL3	MUT	SLC20A1
AFTPH	FN1	MVD	SLC20A2
AGBL5	FNTA	MXD3	SLC25A37
AGMAT	FOXJ1	MYBPC1	SLC25A39
AGPAT4	FOXK1	MYCL1	SLC26A1
AGRN	FOXO3	MYH9	SLC27A5
AGT	FRAS1	MYL5	SLC2A6
AGXT	FSCN1	MYNN	SLC30A7
AHNAK	FSTL3	MYO1B	SLC33A1
AHSA2	FTH1	MYO1D	SLC34A2
AIM2	FUK	MYO5C	SLC35D2

AK3	FUT5	MYOM1	SLC37A4
AKAP1	FUT6	MYPOP	SLC39A14
AKR1A1	FXR1	NAGLU	SLC39A7
AKR1B1	FXYD2	NAGPA	SLC3A2
AKR1D1	FZD6	NAP1L1	SLC41A2
ALDH7A1	FZD7	NAV2	SLC43A3
ALG10	FZD9	NBN	SLC44A1
ALG11	G0S2	NBR1	SLC46A3
ALG6	G3BP1	NCF4	SLC48A1
ALMS1	GABARAPL2	NCOA4	SLC5A1
ALOX5	GADD45A	NDRG4	SLC5A10
ALOX5AP	GADD45G	NDST1	SLC6A14
AMBRA1	GAK	NDUFA2	SLC8A1
AMIGO2	GAMT	NDUFAF1	SLC9A8
AMOTL2	GAPDH	NDUFB7	SLC9A9
AMPD3	GATA4	NDUFS7	SLMAP
ANKRD1	GBA	NDUFV1	SMAD3
ANKRD2	GBAS	NDUFV2	SMARCA1
ANO9	GCDH	NECAP2	SMARCA4
ANP32E	GCH1	NEDD9	SMARCC2
ANXA2	GCLC	NEK6	SMCHD1
ANXA4	GCS1	NEK8	SMG7
ANXA5	GDAP1	NEURL2	SMPD2
ANXA8L2	GDE1	NEURL4	SNAP23
AOF1	GDF15	NFAT5	SNAPC4
AP2B1	GDPD5	NFE2	SNRPE
AP4E1	GFAP	NFE2L3	SNX15
APOA1	GGA1	NFIB	SOD1
APOA2	GGCX	NFKB1	SOD2
APOA5	GGT1	NFKBIB	SORL1
APOB	GHRHR	NIACR2	SP3
APOC3	GHRL	NIN	SP5
APOF	GIMAP8	NINJ1	SPATA5
AQR	GJC1	NIPSNAP1	SPINT3
ARFGAP3	GK	NLRC4	SPPL2A
ARFGEF2	GLCE	NMNAT1	SPR
ARG1	GLT8D4	NOD2	SPSB1
ARL14	GLTPD2	NOL11	SPSB4
ARL2BP	GMDS	NOLC1	SPTBN1
ARL4C	GMNN	NOP16	SQSTM1
ARL5B	GNA15	NOTUM	SREBF2
ARL6IP5	GNAI2	NPB	SRP14
ARNTL2	GNB1	NQO1	SRPK1
ARPC5	GNG12	NQO2	SSB
ARRB1	GNPAT	NR1I3	SSBP3
ART4	GOLT1A	NRARP	SSH1
ASB6	GOLT1B	NRAS	SSSCA1
ASCL2	GORASP2	NRBF2	SSTR2

ASNSD1	GP1BA	NSFL1C	ST3GAL3
ASS1	GPC6	NSL1	ST5
ATAD2B	GPR126	NSMCE1	ST7
ATG4B	GPR137	NT5DC3	STAG1
ATG7	GPR142	NTAN1	STAP2
ATOH8	GPR176	NUAK1	STARD3
ATOX1	GPR37L1	NUAK2	STARD3NL
ATP5G1	GPR44	NUDT6	STARD4
ATP5I	GPR88	NUP153	STAT5A
ATP6V1E2	GPR97	NUP35	STC2
ATP7B	GPRC5A	NUP54	STEAP2
AURKB	GPX1	NUP93	STOML1
AZIN1	GREB1	NUSAP1	STX4
B2M	GRHL1	NXT2	STXBP5
B4GALT1	GRIPAP1	OAS3	SULT1A2
B4GALT5	GRN	OGFOD2	SULT1C4
BAD	GSTO1	OIP5	SUPT4H1
BAIAP2L1	GTPBP2	OLR1	SUPV3L1
BAT2D1	GUCY1B2	OPA3	SVIL
BATF	GULP1	OR4K5	SYDE1
BAX	GYPC	OR5B3	SYNGR2
BAZ1A	H1FX	OR5H1	SYS1
BBC3	H2AFY2	OR7G3	SYT12
BCAT1	H3F3B	ORAI1	SYT8
BCL2L1	H6PD	ORM1	TACSTD2
BCL3	HARBI1	ORMDL3	TAGLN2
BCL6	HAVCR1	OSGIN1	TANK
BCL7A	HBS1L	OTC	TAP1
BET3L	HCN4	OVOS2	TAPBP
BHLHE40	HDGFRP3	P2RY5	TATDN3
BID	HDHD3	PACS1	TBC1D22B
BIRC2	HEATR2	PAF1	TBCA
BIRC3	HECTD1	PAK1	TBL1X
BMP2K	HEG1	PALM2-AKAP2	TBL1XR1
BNIP1	HES1	PAN2	TBP
BNIP3	HIF1AN	PANK1	TBX4
BOLA1	HIGD2A	PANX1	TBX6
BPHL	HINT1	PAPSS2	TCEA2
BRAF	HIST1H4I	PAQR8	TCEB1
BRE	HIST1H4J	PARP1	TCF7L2
BRMS1L	HIST1H4K	PARP12	TERF2IP
BRWD2	HIST4H4	PBX2	TESK2
BSDC1	HIVEP1	PBX4	TESSP2
BTG3	HIVEP2	PCBP3	TEX101
BTG4	HLA-A	PCK1	TFE3
BTN2A1	HLA-DQB1	PCTK2	TGFBR3
BTN2A2	HMGXB4	PDCD1LG2	TGIF2
BTN2A3	HNF1A	PDCD2L	TGM2

BTNL9	HNF1B	PDCD5	THAP4
C1D	HNMT	PDCD7	THEM2
C1RL	HNRNPC	PDE4A	TIFA
C1S	HNRNPL	PDE4DIP	TIGD2
C3	HOMEZ	PDE6D	TINAG
C4BPB	HOOK3	PDE6G	TINAGL1
C9	HOXD1	PDE8A	TINF2
CACHD1	HOXD10	PDGFC	TIPARP
CACNG1	HOXD11	PDLIM4	TJP2
CALML5	HOXD12	PERLD1	TK1
CAMK2D	HOXD13	PEX13	TLCD1
CAP2	HPD	PEX26	TLE1
CAPG	HPN	PFDN4	TLE4
CARHSP1	HPS4	PFN1	TLN1
CASP10	HS3ST6	PFN2	TLR2
CASP4	HSD11B1	PGBD5	TLR6
CCBL2	HSPA1A	PGC	TM9SF2
CCDC115	HSPA1L	PGLYRP2	TMCC2
CCDC76	HSPA4L	PGM3	TMEFF1
CCL16	HSPC159	PGPEP1	TMEM106A
CCL20	HSPG2	PHACTR2	TMEM115
CCL21	HTATSF1	PHF1	TMEM132A
CCL4	HTR2B	PHF10	TMEM140
CCNB1IP1	HTRA4	PHKG2	TMEM141
CCNC	HYAL3	PHLDA3	TMEM165
CCNG1	ICAM1	PHYH	TMEM189
CCNG2	ICAM2	PI3	TMEM19
CCRN4L	ICAM4	PIGV	TMEM205
CD209	ICK	PIK3CD	TMEM217
CD38	IDH1	PIM1	TMEM43
CD44	IFIH1	PIM3	TMEM63B
CD48	IFIT3	PKM2	TMEM69
CD70	IFLTD1	PLA2G2D	TMEM77
CD74	IFNAR1	PLA2G2E	TMEM98
CD82	IFNAR2	PLA2G4C	TMPRSS9
CDK6	IFT20	PLA2G6	TMTC2
CDKN2C	IGF2	PLAU	TNFa
CDYL	IGF2BP1	PLCB3	TNFAIP1
CEBPB	IGF2R	PLEC1	TNFAIP2
CEBPG	IGFALS	PLEKHH3	TNFAIP3
CENPQ	IGFBP1	PLK3	TNFRSF10B
CEP135	IGFBP7	PLXNB1	TNFRSF1B
CETP	IK	PLXNB2	TNFRSF4
CFB	IKBKE	PMAIP1	TNFRSF6B
CFL2	IKZF3	PNPLA6	TNFRSF9
CFLAR	IL13	PNRC2	TNFSF14
CGN	IL17RB	POLRMT	TNIP1
CGNL1	IL1A	POMGNT1	TOB2

CGRRF1	IL1F8	POP4	TOMM5
CHCHD4	IL1RN	PPAPDC1B	TP53
CHD6	IL20	PPFIBP2	TP53I3
CHDH	IL23A	PPIL6	TPCN1
CHMP1B	IL3RA	PPP1R10	TPI1
CHMP4B	IL4I1	PPP1R14D	TPK1
CHRNA10	IL7	PPP1R15A	TPMT
CHST13	IL7R	PPP1R15B	TPT1
CHST4	IL8	PPP1R9B	TRAF1
CIB4	IMP4	PPP3CC	TRAF2
CITED2	INF2	PPP4R1L	TRAF3
CKAP4	INHBC	PPYR1	TRAF4
CKS2	INHBE	PRDX2	TRAPPC10
CLCN6	INO80B	PRELID1	TRAPPC3
CLDN16	IP6K3	PRM1	TRIB1
CLEC4E	IQGAP2	PRMT1	TRIB3
CLGN	IRAK2	PRND	TRIM16L
CLIC4	IRAK3	PRPF3	TRIM21
CLIP2	IRF1	PRPF40B	TRIM25
CLK4	IRF2BP2	PRPF6	TRIM27
CLTB	IRF5	PRRG4	TRIM31
CMTM6	IRF6	PSMA6	TRIM41
COASY	IRX4	PSMB1	TRIM44
COL7A1	ISG20	PSMB9	TRIM47
COQ10B	ISG20L2	PSME1	TRIM67
COQ6	ISM1	PSME2	TRIM8
CORO1C	ITFG1	PTBP1	TRNP1
CORO2B	ITGAD	PTER	TRPM7
COX4I1	ITGAE	PTGES	TRPV2
COX4NB	ITGAM	PTGR2	TRPV3
CP	ITGB2	PTHLH	TSEN2
CP110	ITGB6	PTK6	TSGA10
CPAMD8	ITGB8	PTP4A2	TSKU
CPLX2	ITIH1	PTPN1	TSNAXIP1
CPSF3	ITIH2	PTTG1IP	TSPAN11
CPT2	ITPKC	PTX3	TSPAN14
CRADD	ITSN2	PUS10	TSPAN15
CRAMP1L	IVD	PVR	TSPAN31
CREB3	IWS1	PXK	TTC12
CREB5	JAK3	QKI	TTC21B
CRTC2	JAM2	QTRT1	TTC22
CRY2	JARID2	QTRTD1	TTC23
CRYZ	JDP2	RAB11B	TTC27
CSAD	JUB	RAB34	TTC32
CSF1	JUNB	RAB37	TTC7A
CSF3	KARS	RAB42	TTPA
CSNK1G2	KBTBD7	RAC1	TTRAP
CST5	KCNJ13	RAD51L1	TUBA1C

CSTB	KCNN3	RAD52	TUSC4
CSTF2	KCNQ4	RANBP10	TUT1
CTDSP1	KCTD11	RANBP2	TWISTNB
CTGF	KIF20B	RANGAP1	TYK2
CTHRC1	KIF2C	RAP1A	TYMP
CTPS	KIF3C	RAP2C	TYW3
CTSA	KIFC1	RAPGEF2	UBD
CTSC	KLC1	RAPH1	UBE2H
CTSS	KLF10	RARS	UBE2O
CXCL1	KLF9	RASA2	UBE2W
CXCL10	KLHL38	RASL12	UBE2Z
CXCL2	KREMEN2	RASSF10	UBQLN4
CXCL3	KRT32	RASSF2	UEVLD
CXCR6	KRT6B	RAX	UGT2A3
CYB5R2	KRT75	RB1	UNC119
CYB5R3	KRTAP2-4	RBKS	UNC84B
CYBA	KRTAP3-1	RBM15B	UPK3A
CYCS	KRTAP3-2	RBM19	UQCRB
CYP27A1	KTELC1	RBM28	UQCRC2
CYP27B1	KYNU	RBM39	UQCRHL
CYP4F11	L3MBTL4	RBM9	USP12
CYP4F8	LACTB	RBMS3	USP13
CYP8B1	LAD1	RBX1	USP16
DARS	LAMB3	RCOR3	USP3
DCBLD2	LAMC2	RDH16	USP37
DDX39	LAPTM4A	RELA	USP49
DDX58	LARP4	REM1	USP54
DENND5A	LARP6	REPIN1	USP7
DET1	LASS4	REPS1	USPL1
DHCR24	LBP	RERE	UVRAG
DHFR	LBX2	RETSAT	UXS1
DHRS7B	LCN2	RFX2	VASN
DHX33	LCP2	RFX5	VDAC1
DIAPH2	LCTL	RFXANK	VDR
DICER1	LDHAL6B	RGL1	VEZF1
DLD	LDLRAD1	RHBDL2	VMO1
DNAH1	LEFTY1	RHOF	VNN3
DNAJB2	LFNG	RHOV	VPS16
DNAJC10	LGALS1	RIN1	VPS18
DNAJC2	LGALS8	RIPK2	VPS28
DNAJC5	LGR5	RNASE8	VPS37C
DOCK10	LIMD2	RNASEK	VSNL1
DOT1L	LIMK2	RND1	VTA1
DPP4	LIMS1	RNF114	VTN
DPP9	LINCR	RNF126	WAC
DR1	LIPT1	RNF135	WDR36
DRAP1	LMAN1	RNF144A	WDR75
DTX4	LMBRD2	RNF149	WDTC1

DUSP11	LPGAT1	RNF151	WHSC2
DUSP22	LPXN	RNF170	WRAP53
DUSP5	LRCH1	RNF181	WWC1
DUSP6	LRCH3	RNF186	XAF1
DYRK3	LRG1	RNF207	XKR7
DZIP3	LRRC1	RNF24	YY1AP1
E2F6	LRRC59	RNF31	ZBBX
EBF4	LRRC61	RNMTL1	ZBTB1
EBI3	LRRFIP2	ROBLD3	ZBTB17
ECT2	LSS	ROBO4	ZBTB25
EDAR	LTA	ROR1	ZBTB26
EDC4	LTB	RPGRIP1	ZC3H10
EDEM3	LTBP2	RPH3AL	ZC3H12A
EFHD2	LY75	RPL23	ZC3H12C
EFNA1	LY86	RPL27A	ZC3H12D
EGR1	LY96	RPL5	ZC3H7A
EHD1	LYRM1	RPRD2	ZC3H7B
EIF1	LYRM4	RPS16	ZCCHC8
EIF2B3	LZIC	RPS2	ZDHHC18
EIF3E	MAD2L1BP	RPS3	ZF6
EIF4A2	MAFF	RPS5	ZFAND3
EIF4EBP2	MAGOHB	RPS6KB2	ZFHX3
EIF5A	MAL2	RPUSD4	ZFP36L1
EIF6	MAML2	RQCD1	ZFP42
ELF4	MAN1A1	RRAD	ZFP91
ELF5	MAN2C1	RRAS	ZKSCAN3
ELL2	MAP2K2	RRBP1	ZMIZ2
ELMO2	MAP3K2	RTTN	ZNF101
ELMOD3	MAP4	RXRB	ZNF192
EME1	MAP4K4	S100A10	ZNF2
EMILIN1	MAPK14	SAA1	ZNF223
ENC1	MAPK6	SAA2	ZNF24
EPHA2	MARCKS	SAA4	ZNF267
EPRS	MBIP	SAE1	ZNF3
EPS8	MCCC2	SALL4	ZNF317
ERGIC1	MCHR1	SAPS3	ZNF318
ESPL1	MCM2	SARM1	ZNF323
ETFA	MDM2	SASS6	ZNF335
ETFB	MED18	SAT1	ZNF33B
ETFDH	MED29	SBK1	ZNF354B
ETV6	MEF2B	SBNO2	ZNF426
EVX2	MEFV	SC65	ZNF436
EXTL2	MEIS1	SCAMP3	ZNF44
EZH1	MET	SCARA3	ZNF500
F11	MFSD10	SCARA5	ZNF546
F2	MFSD4	SDCCAG10	ZNF554
F3	MGLL	SDR9C7	ZNF605
FABP1	MGRN1	SEC16A	ZNF670

FAM110A	MGST2	SEC22B	ZNF687
FAM111A	MIA2	SEC24A	ZNF688
FAM113A	MICAL2	SEH1L	ZNF697
FAM120B	MKKS	SELK	ZNF699
FAM129A	MKNK2	SEMA4G	ZNF7
FAM150B	MLL5	SENP3	ZNF706
FAM151A	MLLT6	SEPP1	ZNF718
FAM36A	MMADHC	SEPSECS	ZNF740
FAM69A	MMD	SERINC4	ZNF747
FAM82B	MME	SERPINA11	ZNF749
FAM8A1	MMP2	SERPINA12	ZNF764
FARS2	MMP9	SERPINA6	ZNF768
FBLN2	MOBKL2C	SERPINC1	ZNF781
FBRS	MOGAT2	SERPIND1	ZNF799
FBXL19	MORC3	SERPINE1	ZNFX1
FBXO15	MRAS	SERPINF2	ZNRF1
			ZSWIM4

qPCR primers

Target gene	Forward (5'- 3')	Reverse (5'- 3')
DR4	CCACCACCAGCTAGAGTACAT	GGAAGAGCCCCACACTTT
DR5	AAGACCCTTGTGCTCGTTGTC	GGGCTGGACCTCTTTTGTTGT
BID	CCTACCCTAGAGACATGGAGAAG	TTTCTGGCTAAG CTCCTCACG
BAD	CCCAGAGTTTGAGCCGAGTG	CCCATCCCTTCGTCGTCCT
FLIP	GACAGAGCTTCTTCGAGACAC	GCTCGGGCATACAGGCAAAT
RELA (p65)	CTGCCGGGATGGCTTCTAT	CCGCTTCTTCACACACTGGAT
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

Table S-3.2 Primers for realtime PCR (listed in the 5' to 3' direction).

Chapter 4: Conclusions

4.1 Summary of main findings

The goal of this thesis was to understand the role of intra- and extra-cellular modulators of NF-κB. To this end, I have shown that intracellular proteins that interact and modify NF-κB affect its gene-specific regulation and extracellular stimuli that activate NF-κB in a context-dependent manner greatly influence cellular responses.

In chapter 2, I used affinity purification and mass spectrometry to identify proteins that interact with human p65/RELA, one of the most abundant components of the NF-κB transcription factor. Follow-on co-immunoprecipitation experiments confirmed the interaction (direct or indirect) of the serine/threonine kinase STK38 and its close homologue STK38L with p65. shRNA-mediated knockdown of both STK38 and STK38L reduced NF-κB –mediated transactivation of TNF-α-responsive genes such as LTB and TNF as well as binding of p65 to LTB and TNF promoters. In vitro kinase assay showed that p65 is an STK38/38L substrate and that phosphorylation of p65 at S356 site is necessary for full LTB and TNF induction in vivo.

In chapter 3, I examined the pro-death and pro-survival potential of several human liver cell lines in response to pro-inflammatory cytokine treatments. I showed that pro-inflammatory cytokines had different effects on the apoptotic outcomes of these cell lines when cells were pre-treated with either TNF-α or IL-1α followed by TRAIL. The observed behaviors were very context (cell type, stimulus and time)-dependent. I then demonstrated that the NF-κB pathway was responsible for the diverse apoptotic outcomes induced by pro-inflammatory cytokines TNF-α and IL-1α as introduction of a dominant negative inhibitor of NF-κB into these cells effectively blocked such outcomes. To investigate how activation of NF-κB gave rise to diverse phenotypic outcomes, I took several approaches. I first used ChIP-Seq data to obtain promoters' regions bound by the NF-κB's p65 subunit in response to TNF-α. qPCR and Western blotting

were performed to measure changes in expression of candidate genes. Cells with pro-survival behaviors in response to TNF-α and IL-1α were found to do so by upregulating p65 and FLIP and downregulating DR4 and DR5. The upregulation of p65 and FLIP is more immediate, suggesting that this event is likely responsible for the early pro-survival phenotype whereas the downregulation of DR4 and DR5 occurs after a prolonged treatment of pro-inflammatory cytokines, implying that this event is more important in determining the late pro-survival behavior. For cells that exhibit the pro-death response, upregulation of several apoptotic proteins including BID and BAD appears to play a contributing role.

4.2 Relevance of this work

4.2.1 New mediators of NF-κB in inflammation

NF-κB family proteins have been found to play important roles in many aspects of the immune system during differentiation of immune cells, development of lymphoid organs and during immune activation (Hoffmann and Baltimore, 2006). Research on the biological function of NF-κB has largely concentrated on the mechanisms of signaling that lead to NF-κB activation (Wan and Lenardo, 2010). However, it has been recognized that binding of NF-κB complexes to target DNA might not be sufficient for full gene transcription. Emerging evidence suggests that additional events are required. Like many pleiotropic transcription factors such as p53, NF-κB activity is now known to be regulated by posttranslational modifications as well as co-activators and co-repressors (Natoli and De Santa, 2006).

Dysregulation of NF-κB has been implicated in many inflammatory diseases such as rheumatoid arthritis, asthma, inflammatory bowel diseases, liver fibrosis and cirrhosis (Baud and Karin, 2009; Sun and Karin, 2008). Most current drugs that inhibit NF-κB suffer significant side

effects due to non-selectivity. The ability to effectively target NF-κB for therapeutic intervention requires an understanding of the intricate relationships between NF-κB signaling pathways and the variability in the expression pattern of NF-κB-regulated genes. This involves the identification of new regulatory components of the NF-κB pathway that regulate selectivity in the activation of NF-κB transcriptional programs.

Both TNFα and LTβ belong to the TNF superfamilies of ligands (Balkwill, 2006; Schneider et al., 2004). While TNFα activates NF-κB primarily via the canonical pathway, LTβ does so via the non-canonical pathway. The lymphotoxin system is involved in the development of secondary lymphoid organs, in the formation of ectopic lymphoid structures and in the organization of lymphoid microenvironments (Gommerman and Browning, 2003). TNF-α is, on the other hand, involved in the maintenance and homeostasis of the immune system, inflammation and host defense. Together, both cytokines and their corresponding signaling systems play an important role in the development and function of the immune system.

In this work, two additional NF-κB's coactivators, STK38 and STK38L were identified. These two kinases were shown to modulate the expression of TNFα and LTβ. Overproduction of TNF is the main cause of many inflammatory diseases. Most inhibitors of TNF synthesis work by blocking upstream components of NF-κB's signaling pathway such as IKK. However, blocking NF-κB completely can have side effects as this transcription factor is involved in many important cellular functions. An alternative approach is to selectively inhibit TNF production without too much interference with NF-κB's functions. Targeting STK38 and STK38L instead might help accomplish this.

4.2.2 The importance of contexts in understanding cellular responses

NF-κB is a pleiotropic transcription factor that involves in regulating the expression of many genes. NF-κB-dependent genes control the inflammatory and immune responses, programmed cell death, cell proliferation and differentiation. A variety of stimuli can activate NF-κB via several main groups of receptors such as the TNF receptors family, the pattern-recognition Toll like receptors (TLR), the IL-1 receptors and the antigen receptors (Vallabhapurapu and Karin, 2009). These receptors can potentially activate not only different members of the NF-κB family but also other intracellular signaling and enzymatic proteins, which together results in the induction of a unique gene transcription program dependent on NF-κB.

While NF-κB signaling pathways activated by various stimuli are very complex, a single NF-κB pathway induced by a single ligand is not simple either. As demonstrated in this work, the same TNFα or IL-1α stimulation can have very different effects on cellular responses including the programmed cell death. While in some cells, TNFα and IL-1α confer pro-survival advantage against TRAIL killing, in other cells, the effect is the opposite. In addition, duration of TNFα and IL-1α treatments can also have distinct outcomes. Since activation of NF-κB is necessary, this implies that different NF-κB gene regulation programs are responsible for such diverse behaviors. This shows that NF-κB responses are very context-dependent, be it cell type, stimulus or time. In order to understand how NF-κB functions, it is therefore important to look at the context surrounding its activation. Although this undoubtedly increases the complexity of the NF-κB signaling pathway, it also provides us with valuable information about different NF-κB's gene expression programs, from which insights into how NF-κB's target genes are regulated and discovery of new regulatory mechanisms can be gained. In this work, applying a context-dependent approach, several TRAIL resistance mechanisms were proposed. The first mechanism

involves upregulation of FLIP and the second mechanism involves downregulation of TRAIL receptors DR4 and DR5. While FLIP upregulation is very fast, downregulation of DR4 and DR5 happens at a much later time. Some cells can effectively activate both mechanisms with the first one as the early response to pro-inflammatory cytokines and the second one as the late response. Also, upregulation of NF-κB's p65 subunit appears to play a key role in changes in FLIP and DR4 and DR5 protein levels. While p65 directly up-regulates FLIP, it indirectly down-regulates DR4 and DR5 by possible turning on a ubiquitin-mediated degradation of these receptors.

4.2.3 Pro-death role of NF-κB

NF-κB is mostly known for its pro-survival function as p65 deficient cells undergo apoptotic cell death upon treatment with TNFα due to inadequate pro-survival gene transcription (Beg and Baltimore, 1996). In addition, p65 knockout cells lead to embryonic lethality accompanied by massive apoptosis in the embryonic liver due to TNFα exposure (Beg et al., 1995). Also, in response to other stimuli such as ionizing radiation and chemotherapeutic drugs, p65 appears to have an anti-apoptotic effect (Barkett and Gilmore, 1999).

While the pro-survival role of NF-κB has been extensively characterized, little is known about its pro-death function and the mechanism by which NF-κB mediates a pro-death response is not well characterized. Recent studies suggest that the pro-death function of NF-κB is due to its repression of pro-survival genes such as Bcl-2 and XIAP and induction of pro-death genes namely TNFα and Fas ligand (Ho et al., 2011).

In this work, in response to pro-inflammatory cytokine TNFα and IL-1α stimulation followed by TRAIL, Hep3B cells were shown to exhibit a pro-death phenotype. NF-κB was demonstrated to be responsible for this outcome as blocking its activation prevented the pro-

death behavior. However, the pro-death function of NF-κB in this cell line was not due to repression of pro-survival genes as their expression could still be detected. It is mainly the upregulation of pro-death genes that contributes to the observed response. Interestingly, the type of pro-death genes induced by NF-κB in Hep3B cells is rather different from what was shown in other studies which suggested that the pro-death role of NF-κB was due to TNFα and FasL synthesis. In fact, neither TNFα nor FasL alone was able to kill Hep3B cells. Taken together, this work not only described the pro-death function of NF-κB but also suggested a different mechanism for such function.

4.2.4 Inflammation and cancer

It has long been suspected that there is a link between inflammation and cancer based on observations that virtually every tumor is surrounded by immune cells (Hanahan and Weinberg, 2011). Such immune responses were originally thought of as an attempt by the immune system to eradicate tumors, which is true in some cases. However, tumor-associated inflammation has been shown to have an unanticipated effect of enhancing tumorigenesis and progression. Inflammation can cause the release of cytokines to the tumor microenvironment that can facilitate proliferation, survival, angiogenesis, invasion, metastasis, and activation of epithelial-mesenchymal transition.

Elevated NF-κB activity has been observed in many solid tumors. In such cancers, NF-κB activation is a result of the underlying inflammation or the consequence of formation of an inflammatory microenvironment during malignant progression. Genes regulated by NF-κB can affect all aspects of cancer development including inhibition of apoptosis, stimulating cell proliferation as well as promoting a migratory and invasive phenotype. Thus, NF-κB is believed

to play a critical role in the intricate relationship between inflammation and cancer (Karin, 2009; Karin et al., 2002).

While the important role of inflammation in tumorigenesis has now been recognized, it is still not easy to study this relationship in vivo. An alternative approach is therefore to use an in vitro cell culture system to explore how inflammation affects various aspects of cell behaviors. In this work, such in vitro approach was used to study the role of pro-inflammatory cytokines in the apoptotic responses of human liver cells. Using this type of approach, diverse apoptotic behaviors were observed. This suggests that inflammation has different effects on different cells and that these diverse behaviors reflect the complex and distinct gene transcription programs regulated by NF-κB. Although, this in vitro context-dependent approach was applied to investigate only the cell death and survival aspects induced by inflammation, there is no reason why such approach cannot be used to explore other inflammation-induced pathways.

Given the role of inflammation in cancer and the fact that tumor cells respond to inflammation differently, one has to take all of these into consideration when it comes to cancer treatment. An example of this involves TRAIL. This promising anti-cancer drug has attracted considerable attention owing to its selective killing of tumor cells while normal cells were largely protected (LeBlanc and Ashkenazi, 2003). In animal models, treatment with TRAIL by itself or in combination with other drugs resulted in suppression of tumor xenografts without causing systemic toxicity (Kelley and Ashkenazi, 2004). Clinical trials using TRAIL have been reported for patients with non-small-cell lung cancer, non-Hodgkin's lymphoma and colorectal carcinoma (Fischer and Schulze-Osthoff, 2005). Despite its tumor specific killing, some tumors can survive TRAIL treatment, which presents a major barrier for the development of efficient therapies (Kurbanov et al., 2007; Morizot et al., 2010). Resistance to TRAIL has been suggested

to be due to increased levels of antiapoptotic proteins such as c-FLIP, Bcl-2, Bcl-xL and inhibitors of apoptosis proteins, all of which are regulated by NF-κB.

4.3 Future work

In this work, STK38 and STK38L were demonstrated to bind and phosphorylate NF-κB's p65 subunit in vitro. While these two kinases have been studied to a great extent in yeasts, little is known about their substrates in mammalian cells (Hergovich et al., 2008). It will therefore be important to identify additional substrates of these kinases. Creating knock-in cells that expressed active-site STK38/38L mutants that can recognize an ATP analog (using methods developed by Shokat and colleagues) (Hindley et al., 2004) and using mass spectrometry to identify modified substrates would be one way forward.

STK38 and STK38L were shown to modify p65 on several residues, one of which is S356. Since this is a novel phospho-acceptor site of p65, in order to study the phosphorylation of this site in vivo, it is important that a phospho-specific antibody for p65-S356 is produced. Also, modification of p65 at S356 was shown to affect gene transcription of some NF-κB-dependent genes including TNF and LTB. There are probably more genes whose expressions are regulated by this modification. So it will be interesting to identify these additional genes by creating a knock-in cell line carrying a serine to alanine mutation and using gene expression profiling to screen for such genes. Also, since NF-κB can be activated by a variety of stimuli, it is likely that different genes are affected by p65-S356 phosphorylation in response to different treatments.

In the study of how inflammation affects TRAIL-induced cell death behaviors, several mechanisms were proposed. Mechanisms resulting in TRAIL resistance involve upregulation of FLIP and downregulation of DR4 and DR5. To confirm that FLIP plays an important role,

knocking down of this protein is required. As for downregulation of DR4 and DR5, it is obvious that this process directly mediates TRAIL resistance. However, what is not obvious is how NF-κB activation leads to degradation of death receptors. One possible explanation is due to expression of a ubiquitin ligase. Work is underway to identify proteins recruited to DR4 and DR5 in response to TNFα and IL-1α stimulation that have a ubiquitin ligase function.

While activation of the NF-κB pathway by pro-inflammatory cytokines results in expression of pro-survival genes, in some case, this pathway can induce expression of pro-death genes. Since little is known about the pro-death role of NF-κB, it will be interesting to explore this further. While the most likely explanation is due to expression of pro-apoptotic proteins such as BID and BAD, it is also possible that regulation at the receptor level can contribute to this phenotypic outcome. To examine how homo- versus hetero-trimerization of TRAIL receptors influences the cell death response, one can introduce the missing receptor (DR4) into cells that express only one type of death receptor, for example DR5. In fact, it has been suggested that formation of different types of receptor's trimers upon TRAIL stimulation can have different apoptotic outcomes (Lemke et al., 2010).

The NF-κB pathway is known to be involved in activation of many genes. In this work, several genes and proteins with the most likely contribution to the overall apoptotic behaviors of various cell types were shown. However, this does not rule out the involvement of other genes and proteins regulated by NF-κB. A large scale expression profiling of various liver cell lines in response to TNF-α and IL-1α using a Luminex bead-based mRNA measurement approach is currently underway. This method can profile up to 1000 target gene expression per condition and can also process a large number of samples. One hopes that with these types of data, additional

genes and proteins that not only play a role in the apoptosis pathway but also other important cellular processes are discovered.

4.4 Closing thoughts

The transcription factor NF-κB, more than two and a half decades after its discovery, remains an exciting and active area of research. Although NF-κB biology is complex due to many layers of its regulation, we are starting to understand more about the role and function of this transcription factor in various cellular pathways as well as disease states. While it is no doubt that more work needs to be done to uncover new players and mechanisms of gene regulation by NF-κB, I hope that this thesis contributes to furthering our knowledge of NF-κB and benefiting future research in this field.

4.5 References

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